Rapid Quantitative Analysis of Sphingolipids in Seafood Using HPLC with Evaporative Light-Scattering Detection: Its Application in Tissue Distribution of Sphingolipids in Fish

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Abstract: Sphingolipids are ubiquitous in all eukaryotic organisms and known to be essential constituents of cellular membranes. Recently, various physiological functions of dietary sphingolipids, such as preventing cancer, improving skin barrier and contributing to central nervous system myelination have been demonstrated. To characterize the sphingolipids from fish as food components, tissue distribution of sphingomyelin and glycosylceramide (ceramide monohexoside, CMH) in fish were determined in this study. We established a rapid, accurate and effective method for separation, purification and determination of sphingolipids by using high-performance liquid chromatography with evaporative light-scattering detector (ELSD-HPLC). Sphingolipids were extracted and quantified from pacific saury (Cololabis saira). Sphingomyelin in different tissues of Cololabis saira ranged from 2.5 ± 0.2 mg/g to 27.6 ± 2.1 mg/g, the content in brain was the highest, followed by eyes, and CMH contents were less than 23.0 ± 2.4 mg/g in all tissues. These results revealed that fish contained CMH and sphingomyelin as same levels as most of the terrestrial organisms and suggested marine organisms could be used as a potential source of precious and useful complex lipids.

Key words: sphingolipids, sphingomyelin, glucosylceramide, HPLC-ELSD, fish

1 INTRODUCTION

Sphingolipids are ubiquitous in eukaryotic organisms and a few bacteria1. Phospholipids and glycolipids are typically two kinds of sphingolipids as constituent located in the outer leaflet of the plasma membrane2-4. Their hydrolyzed products (ceramides and sphingoid bases) are highly bioactive compounds playing as second messengers that are known to be involved in essentially all aspects of cell regulation, such as cell growth, cell differentiation and apoptosis5-7. Despite of relatively small amounts, sphingolipids are significant components in foods and highly essential for human health8. Dietary sphingolipids have beneficial effects such as preventing cancer9-14, improving skin barrier15 and contributing to central nervous system myelination16.

As constituents of most foods, the major sphingolipids that we ingest daily are sphingomyelin and glycosylceramide (ceramide monohexoside, CMH). Some researchers have summarized the amounts of sphingolipids in several kinds of foods or meal samples17-19. However, constituents of sphingolipids from seafood are not clear yet. Moreover, the previous quantification methods to determine sphingolipids have been costly or time-consuming. In order to classify and quantify sphingolipids in total lipids of seafood, a rapid and effective method is required.

High-performance liquid chromatography (HPLC) is one of the most popular tools for analysing lipid classes. Besides, the evaporative light-scattering detector (ELSD) for HPLC which is insensitive to the mobile-phase solvents and thus allows direct quantification, has been successfully applied to analyze glycolipids in edible plants17, yeast20 and other foodstuff21, 22. The aim of this study was to develop an optimal HPLC-ELSD condition for separation, purification and determination of sphingolipids. By using this convenient and rapid binary gradient system without time-consuming pretreatment of the Folch’s lipid extract23, we classified and determined sphingolipids from several tis-
sues of fish.

2 MATERIAL AND METHODS

2.1 Materials

Ceramide, cholesterol, glucosylceramide from human spleen (ceramide monohexoside), and sphingomyelin from bovine brain were purchased from Sigma (USA). Hexane, methanol, chloroform and 2-propanol were purchased from Nacalai Tesque (Kyoto, Japan), of all were HPLC grades. Other used regents were of analytical grade.

2.2 Sample preparation

Pacific saury (Cololabis saira) (n = 3) was purchased from a local supermarket (Kyoto, Japan). After washing with saline, samples were autopsied to acquire different tissues, then lyophilized and milled. Total lipids were extracted by Folch’s method and were saponified with 0.4M KOH in methanol at 38°C for 2h to remove glycerolipids. The alkali-stable lipid fractions were applied to HPLC-ELSD. The sphingolipid concentrations were expressed as the mean of a paired analysis of the same sample.

2.3 HPLC-ELSD conditions

The HPLC system consisted of double LC-6AD HPLC pumps (Shimadzu Co., Kyoto, Japan) equipped with vacuum degassers, a 100 μL manual injector (Rheodyne 7725i, USA), a CTO-10Avp column oven (Shimadzu), low-temperature evaporative light scattering detector ELSD-LT II (Shimadzu) furnished air compressor Akpsel-con YC-3F (Yaezakiku-atu Co., Tokyo, Japan) and cooling water circulation system CCA-1111 (EYELA, Tokyo, Japan). The evaporation temperature and pressure for ELSD were kept on 40°C and 350 kPa, respectively. Analytical column was TSKgel CN-80Ts (250 × 4.6 i.d. mm, 5 μm) (TOSOH, Tokyo, Japan), and maintained at 40°C. The mobile phase consisted of hexane–2-propanol (99:1 vol/vol) and chloroform–methanol (60:40 vol/vol) with flow rate 1.0 mL/min (Table 1). It was necessary to re-equilibrate the column with the starting solvent mixture for 10 min prior to the subsequent injection. The photomultiplier sensitivity was adjusted to gain 6.

Table 1 Elution Program Employed for the Binary Gradient System

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

Solvents: (A) hexane-2-propanol (99:1 vol/vol), (B) chloroform–methanol (60:40 vol/vol).

Data were collected by LC solution workstation software (Shimadzu).

Fig. 1 HPLC-ELSD Chromatogram of Standard Lipids. A mixture of cholesterol 1 μg (A), ceramide 1 μg (B), glucosylceramide 10 μg (C) and sphingomyelin 20 μg (D) was injected.

Fig. 2 Calibration Curves of Ceramide Monohexoside and Sphingomyelin in HPLC-ELSD.
3 RESULTS

3.1 HPLC-ELSD of sphingolipids

Four kinds of alkali-stable lipids (cholesterol, ceramide, CMH and sphingomyelin) were clearly and completely separated from each other within 20 min with the developed HPLC-ELSD conditions that we obtained in this study (Fig. 1). The retention times for each kind of standard lipid were 6.0, 9.5, 12.1 and 15.1 min, respectively. With same concentration, the detection sensitivities were quite different among these four lipids. Peak areas of the cholesterol and ceramide were approximately 2 to 3-fold greater than CMH and sphingomyelin. Moreover, the peak height of sphingomyelin was the lowest with same concentrations of these four kinds of lipids.

The calibration curves were run with concentrations from 0.5 to 3 μg of CMH and from 1 to 6 μg of sphingomyelin. For three determinations at different concentrations of sphingolipids, the standard deviation values were within 5% of the mean. Both two calibration curve were expressed as the equation \(y = ax^b\) and the regression correlation co-efficient were above 0.995 (Fig. 2).

3.2 Sphingolipids of pacific saury extract

Figure 3 shows HPLC-ELSD chromatograms of Folch’s extract followed by alkali treatment in different tissues from pacific saury (Cololabis saira). Contents of CMH and sphingomyelin from different tissues are summarized in Table 2. The contents of CMH ranged from 1.4 ± 0.1 to 23.0 ± 2.4 mg/g, approximately 1.2 to 2 times much as CMH in each different tissue. Both these two kinds of sphingolipids were highest in brain, and then followed by eyes.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CMH</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>23.0 ± 2.4</td>
<td>27.6 ± 2.1</td>
</tr>
<tr>
<td>Eye</td>
<td>3.3 ± 0.4</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>Viscera</td>
<td>2.3 ± 0.3</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.9 ± 0.3</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.8 ± 0.2</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Liver</td>
<td>1.4 ± 0.1</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Skin</td>
<td>0.4 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of three-independent experiment conducted in duplicate.

4 DISCUSSIONS

The evaporative light-scattering detector (ELSD) can detect the signals of target substances without affect from
Detection sensitivity of sphingomyelin was lower than other lipids (Fig. 1). By increasing the methanol concentration of mobile phase, the peak corresponding to sphingomyelin becomes sharper and easier to be detected. However, the retention times of the peaks become extremely short, thus makes hard to separate the individual peaks. We optimize the methanol concentration and the elution program as shown in Table 1 for the retention times and peak areas to stay fairly reproducible and to regenerate a reasonable scale.

Two main sphingolipids, CMH and sphingomyelin, from several tissues of fish were determined by the proposed HPLC-ELSD system with a TSK gel CN-80Ts column. We demonstrated that sphingomyelin and CMH are ubiquitous in tissues (Fig. 3 and Table 2). In general, two kinds of CMH having a different monosaccharide head group are present in animals. Glucosylceramide (glucocerebroside) is CMH in which the monosaccharide head group is glucose, meanwhile, galactosylceramide (galactocerebroside) is a type of CMH consisting of a ceramide with a galactose residue at the 1-hydroxyl moiety. However, they could not be distinguished and separated by this method we used here. It has been reported that both of these two kinds CMH exist in fish brains, and glucosylceramide was around 40% of CMH in various marine fish brain but very low in fresh water fish, for which was less than 1% 23. Furthermore, galactosylceramide were two times more than glucosylceramide in rectal gland of spiny dogfish 26. Galactosylceramide might be constituted nearly 50% of CMH in pacific saury.

According to the report by Rombaut et al., sphingomyelin occurs 400-1500 mg/kg in dry matter base of dairy products, including milk, butter and cheese 21, 22. Sphingomyelin in animal sources such as turkey, chicken, ostrich, lamb, hamburger, beef, pork, ham and egg were ranged from 100 to 400 mg/kg, for which was lowest in egg and highest in chicken. It has been reported that contents of sphingomyelin in salmon, herring, cod and plaice were 60-140 mg/kg 27. On the other hand, contents of glycosphingolipids in fish (25-100 mg/kg) were relative higher than in meat (25-65 mg/kg) 29. Sphingomyelin were as 1.2 to 2 times more than glycosphingolipids in each specimen. In the present study, contents of sphingomyelin and CMH in pacific saury (shown in mg/g dry weight in Table 2) confirmed this result, but with higher content of both CMH and sphingomyelin. Fish brain is known to be rich in phospholipids, and in spite of the difference in fish phylogeny and ecology constitutions would be quite different 30.

In conclusion, the proposed system provided a rapid, economic and effective method for separation, purification and determination of sphingolipids from several tissues of fish. Our data also demonstrated that fish contains CMH and sphingomyelin as same levels as most of terrestrial organisms do. And for this reason, marine organism could be used as a potential source of precious and useful complex lipids.

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References


