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Separation and Determination of Functional Complex Lipids from Chicken Skin

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Abstract Complex lipids including sphingolipid and plasmalogens were expected to be used as functional supplement, although their physiological activities have not been fully demonstrated. Although these complex lipids exist voluminously in brain and nervous tissues, hardly any animal resources of these lipids have been used since the outbreak of bovine spongiform encephalopathy. Thus, the chemical composition and concentration method of complex lipids from the skin of mature laying hens, a huge amount of which is wasted every year, has been investigated. Total lipid yield (32 g/100 g) prepared from chicken skin contained 2% complex lipids. Total lipids predominantly consisted of triacylglycerol (TAG), with phosphatidylcholine, sphingomyelin (SM) and phosphatidylethanolamine (PE) generally predominant as complex lipids. PE was primarily plasmalogens (62 mol%), of which arachidonic acid (47.6 mol%) and docosahexaenoic acid (11.2 mol%) were the predominant fatty acids. The component sphingoid base of sphingomyelin was almost totally 4-*trans* sphingenine (sphingosine). The complex lipids were able to be separated from an ethanol extract of minced skin in good yield by solvent fractionation with a hexane/ethanol system. Moreover, highly purified SM (>95 wt%) was prepared by a combination of solvent fractionation and alkaline/acidic hydrolysis from the ethanol extract. Thus, it was shown that culled chicken skin could be a potential resource of the antioxidant phospholipid plasmalogens and human-type sphingolipid.

Keywords Sphingomyelin · Plasmalogens · Complex lipids · Sphingolipid · Phospholipid · Sphingosine · Alkenylether · Chicken skin · Culled chicken

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Introduction

Complex lipids including glycerophospholipids and sphingolipids are important components of the biological membrane. From the aspect of food-functionality of lipids, some complex lipids are also known to have physiological functions. Among glycerophospholipids, plasmalogens have been noted as an antioxidant phospholipid in vitro and in vivo [1–3]. Moreover, uptake of plasmalogens was speculated to prevent the lipid oxidation of vascular epithelial cells, since it was absorbed into the intestinal tract followed by a large increase in the plasmalogen level in blood plasma [4]. However, a source of plasmalogen is not readily available.

Sphingolipids, including ceramide, are abundantly present in animal epidermis [5] or isolated epidermal cells [6], and are major intercellular lipids that maintain the skin barrier function [7, 8]. Dietary sphingolipids such as cerebroside (glucosylceramide) and sphingomyelin were

reported to prevent the early stage colon cancer [9–11] and improve the skin barrier function [12, 13]. Nerve tissues are rich in these complex lipids [14], which have been separated from bovine brain to be used as supplement and cosmetic materials. The outbreak of bovine spongiform encephalopathy has limited the use of animals as a source of cerebroside. Currently, cerebrosides of plant origin are an available functional supplement despite the expensive purification cost [13, 15]. However, sphingolipids of plant origin are mainly composed of sphingoid bases (8-unsaturated bases), distinctive from those of mammals (4-*trans* sphingenine), and have limited bioavailability [16, 17].

In Japan, 130 million mature laying hens are bred every year, of which 90 million hens are culled. The culled chickens are commonly incinerated because of their tough flesh compared to broiler chickens, thus an alternative use for the culled chickens has been called for. In the present study, we investigated the chemical compositions and a simple extraction and concentration method of for removing complex lipids from culled chicken skin. Thus, removal of these lipids would add value to culled chickens.

Experimental Procedures

Materials

Skins were separated from 30 culled chickens (700 days old; breed, Julia) that had been fed a diet of 62% corn, 20% plant oil cake and 6% animal-based feed at the Nansatu Syokucyo Co., Ltd. (Kagoshima, Japan). Briefly, slaughtered chickens were skinned on either side after cutting the back of the chicken with a knife. The skin (approximately 5 kg) was cut into 8 mm pieces using a mincing machine, uniformly mixed and frozen. Standard samples of phosphatidylethanolamine (PE, synthetic dipalmitate), phosphatidylcholine (PC, from egg lecithin hydrogenated), phosphatidylserine (PS, from soy beans) and sphingomyelin (SM, from bovine brain) were purchased from Sigma (St. Louis, USA), and cerebroside (CE, lower spot from bovine brain) was purchased from Doosan Serdary Research Laboratories (Toronto, Canada).

Extraction of Total Lipids and Separation of Complex Lipid Classes

A portion (10 g) of minced skin was extracted twice with 100 ml chloroform-methanol (2:1 v/v) and once with 100 ml chloroform–methanol (1:2 v/v) for 1 h at room temperature, respectively. After filtration using filter paper (Advantec No. 2, Toyo Roshi Kaisha, Japan), the extracts were combined and washed once with water at a ratio of chloroform–methanol–water (8:4:3 v/v/v) [18]. After centrifugation at 1,500*g* for 15 min, the lower phase was concentrated to dryness using a rotary evaporator. The lipids thus obtained were weighed and redissolved in chloroform. Total lipids were applied to silicic acid column chromatography. Neutral and complex lipid fractions were eluted from the column with chloroform and methanol, respectively [19]. Additionally, the complex lipid fraction was applied to silicic acid column chromatography, and PE and CE were eluted with chloroform–methanol (4:1 v/v), PC was eluted with chloroform–methanol (2:1 v/v) and SM was eluted with chloroform–methanol (1:2 v/v) [20]. Then, each fraction was separated by preparative silicic acid TLC (Silica gel 60, 0.25 mm, Merck, Germany) with chloroform–methanol–water (65:25:4 v/v/v), and each compound, made visible by spraying the plate with 0.001% primuline solution followed by UV irradiation, was recovered from the silica gel. Complex lipids were separated and detected by two-dimensional TLC developed in chloroform–methanol–25% ammonium hydroxide (65:35:6 v/v/v) in the first direction and chloroform–acetone–methanol–acetic acid–water (50:20:10:10:5 v/v/v/v/v) in the second direction, and by TLC developed in chloroform–methanol–water (65:25:4 v/v/v). Neutral lipid components were analyzed by silicic acid TLC with hexane–diethylether–acetic acid (80:20:1 v/v/v). Total and complex lipid yields were average from more than three independent experiments.

HPLC Analysis

The complex lipid fraction was analyzed by normal-phase HPLC under the following conditions [21]. Instrument: Shimadzu LC-10AD (Tokyo, Japan); mobile phase: solvent A, chloroform–methanol–triethylamine buffer (pH 3, 1 M formic acid) (87.5:12:0.5 v/v/v) and solvent B, chloroform–methanol–triethylamine buffer (28:60:12 v/v/v); gradient (solvent B%): 0–20 min (0–100%), 20–25 min (100–0%); column: LiChrospher Si60 (column size, 250 mm × 4 mm i.d.; particle size, 5 μm, Merck, Germany); flow rate: 1 ml/min; injection volume: 20 μl; column temperature: 40 °C; detector: Shimadzu ELSD-LT (70 °C, 350 kPa). The amount of each compound was calculated from the calibration curve of each standard compound except for phosphatidylinositol (PI, calculated as PS equivalent). All data are averages of three independent experiments.

Methanolysis of Complex Lipids

To determine the fatty acid components, the complex lipid fraction and phospholipid classes were reacted with 5% hydrochloric acid in methanol at 100 °C for 3 h. After cooling, the fatty acid methyl esters were extracted three times with *n*-hexane after the addition of water. To

determine the sphingoid base component, separated sphingolipids were reacted with 1 M hydrochloric acid in methanol at 70 °C for 18 h [22]. After removing the fatty acid methyl esters three times with *n*-hexane, the lower phase was adjusted to pH 10 by the addition of 4 N aqueous sodium hydroxide. The sphingoid base released was then extracted three times with diethylether after the addition of water.

Acetolysis of Complex Lipids

Acetolysis was used to determine the *sn*-2 fatty acid of plasmalogens [23]. Briefly, plasmalogens with 1-alkenylether bond were converted to 1-acetyl-2-acyl-3-acetylglycerolipids whereas diacylglycerophospholipids were converted to 1,2-diacyl-3-acetylglyceride, which were definitively separated on TLC. Each glycerophospholipid class was reacted with acetic anhydrate/acetic acid (3:2 v/v) at 145 °C for 6 h. After cooling, chloroform–methanol–water (8:4:3 v/v/v) was added to the reaction solution, and the lower phase was separated and dried by evaporation under a nitrogen gas. The crude mixture was subjected to preparative silicic acid TLC with hexane–diethylether (80:20 v/v) to separate 3-acetyl-1,2-diacylglyceride (from diacylphospholipid) and 1,3-diacetyl-2-monoacylglyceride (from plasmalogens), which were methanolyzated as described above.

GC and GC–MS Analyses

Fatty acid methyl esters were analyzed using a Shimadzu GC-2010 (Tokyo, Japan) equipped with FID under the following conditions. Column: CP-SIL 88 (50 m × 0.25 mm i.d., 0.2 μm, Varian, USA); temperature program: 80–160 °C (10 °C/min), 160–220 °C (2 °C/min); carrier gas: nitrogen; gas flow rate: 30 ml/min; detection: 240 °C; injection mode: split. Sphingoid bases were converted to TMS-ether derivatives with hexamethyldisilazane–trimethylchlorosilane–dry pyridine (1:1:1 v/v/v) at 60 °C for 1 h, which were analyzed using Shimadzu GCMS-QP2010 (Tokyo, Japan) under the following conditions. Column: ULBON HR-1 (50 m × 0.25 mm i.d., 0.25 μm, Shinwa Chemical Industries, Japan); temperature program: 150–260 °C (8 °C/min), 260–320 °C (2 °C/min), held at 320 °C for 2 min; carrier gas: helium; detect: EI 70 eV. Fatty acid methyl esters and TMS-ether derivative of sphingoid bases were judged by comparing GC retention times and mass spectrum by GC–MS. Fatty acid composition (mol%) was calculated as peak area/molecular weight. All data are averages of at least three independent experiments, except for data presented in Table 3 (average of duplicate analyses).

Separation of Complex Lipids from Minced Chicken Skin

Methods of extraction and concentration with *n*-hexane and ethanol, an approved food additive, were investigated (Fig. 1), since the neutral lipid fraction was predominantly present in culled chicken skin. Minced chicken skin was treated twice with *n*-hexane (0.5 l/kg) for defatting and then extracted with ethanol (1.5 l/kg) (oily sample A, if concentrated to dryness). The extract was partitioned by the ratio of hexane–ethanol–water (8:4:1 v/v/v). The lower ethanol–water phase was separated and concentrated to dryness using a rotary evaporator (sample B). Phosphorus contents in the lipid samples were determined according to the method of Bartlett [24].

Separation of Sphingomyelin Concentrates from Ethanol Extract of Chicken Skin

The method of separation and concentration of SM, a lipid stable with mild alkali and acid, from complex lipids in culled chicken skin, is shown in Fig. 2. TAG and glycerophospholipids included in the ethanol extract were hydrolyzed with 0.4 N sodium hydroxide in ethanol at 37 °C for 1 h, and then the remaining lysoplasmalogens with an alkenylether bond were hydrolyzed with 50% H_2SO_4 at 37 °C for 1 h. After neutralization with 4 N sodium hydroxide in ethanol, the solvent was fractionated with hexane–ethanol–water mixture as shown in Fig. 1. The lower phase was concentrated to dryness using a rotary evaporator, after removing the solid matter by filtration at 40 °C. In precipitation steps, centrifugation was carried out at 1,500*g* for 10 min at room temperature. Different

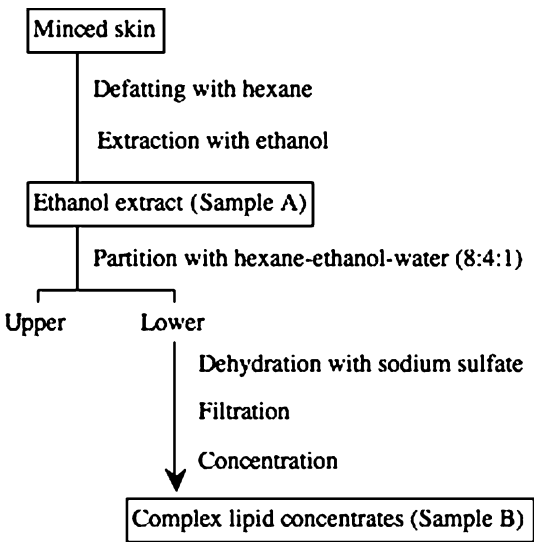

Fig. 1 Procedure for separation of complex lipids from minced chicken skin by ethanol extraction followed by solvent fractionation

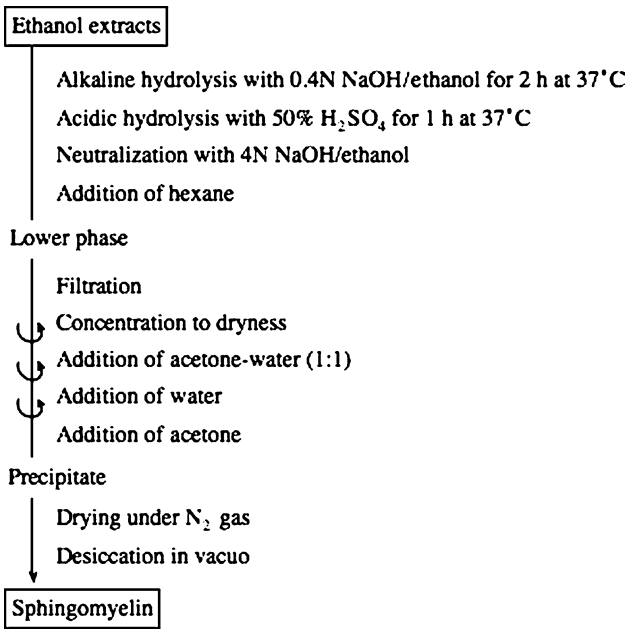

Fig. 2 Procedure for separation of sphingomyelin concentrates from ethanol extract of chicken skin

preparation of complex lipids and sphingomyelin were performed independently.

Results and Discussion

Detection of Complex Lipids in Chicken Skin and Fatty Acid Analysis

The yield of total lipids obtained from chicken skin was 32 g/100 g, which comprised of 98 and 2 wt%, neutral and polar lipid fractions, respectively. The neutral lipid fraction predominantly consisted of TAG, although Wertz et al. [25] reported that cholesterol and wax as well as TAG were abundant in chicken epidermis. This difference in lipid composition may be due to the method by which the chicken skin was obtained and the amount of fatty tissue used in the extraction.

Subsequently, analysis of complex lipids by normal-phase HPLC revealed that PC, SM and PE were 40, 27 and 18 g/100 g total complex lipids, respectively (Fig. 3). The result corresponded to the previous report that the major phospholipids in chicken epidermis were PE, PC and SM [25, 26]. It was thus demonstrated that SM, a functional lipid, was significantly present in skin from culled chickens.

Fatty Acid Composition of Complex Lipids

Seventeen fatty acids from myristic acid (14:0) to tetracosadienoic acid (24:2) were detected, and five species of dimethylacetals were also detected (Table 1). Since

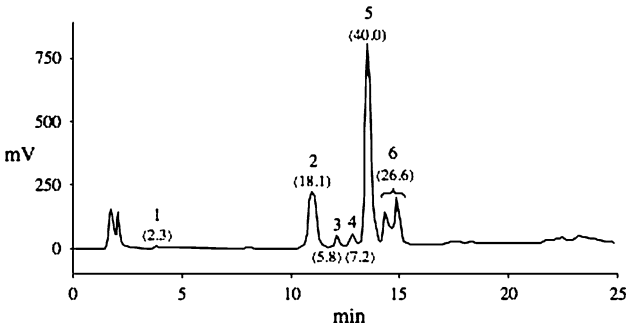

Fig. 3 HPLC of complex lipids in chicken skin. *1* Cerebroside, *2* phosphatidylethanolamine, *3* phosphatidylinositol, *4* phosphatidylserine, *5* phosphatidylcholine and *6* sphingomyelin. *Values* in *parentheses* indicate the weight percent of each lipid expressed as means by triplicate analyses

Table 1 Compositions of acyl and alkenylether moieties in complex lipid fraction

Moieties	Mol%[a]
Acyl	
14:0	0.8 ± 0.4
16:0	27.0 ± 0.4
16:1	1.1 ± 0.3
18:0	18.1 ± 0.9
18:1(n-9)	15.2 ± 0.2
18:1(n-7)	2.8 ± 0.4
18:2(n-6)	9.6 ± 0.3
20:0	0.7 ± 0.1
20:4(n-6)	12.1 ± 0.3
22:0	2.2 ± 0.3
22:3(n-6)	1.5 ± <0.1
22:5(n-3)	2.0 ± <0.1
22:6(n-3)	4.5 ± 0.2
23:0	0.2 ± <0.1
24:0	0.6 ± <0.1
24:1(n-9)	1.4 ± 0.1
24:2(n-6)	0.2 ± <0.1
Alkenylether (dimethylacetal)	
16:0	40.5 ± 0.4
18:0	45.4 ± 0.8
18:1(n-9)	8.8 ± 0.9
18:1(n-7)	2.2 ± <0.1
20:0	3.1 ± 0.1
Acyl:Alkenyl	89:11

[a] Values are the means of more than triplicate analyses

dimethylacetals are released from acidic hydrolysis of the alkenylether bond, the existence of this compound indicates that part of the phospholipids must be plasmalogens. The molar ratio of acyl and alkenylether in the complex lipid faction was 89:11, indicating that proportion of

plasmalogens in complex lipid fraction was approximately 22 mol%, since plasmalogens have one alkenylether and one acyl moiety. This equals the proportion of plasmalogens in bovine brain phospholipids [27].

The major fatty acids in the complex lipid fraction were 16:0, stearic acid (18:0) and oleic acid (18:1n-9). Moreover, highly unsaturated fatty acids (HUFAs), including arachidonic acid (20:4n-6) and docosahexanoic acid (22:6n-3), and long chain fatty acids with a carbon length more than 24, were also detected. Two species, 18:0 and 16:0, comprised the majority of the dimethylacetals.

Chemical Composition of PE and PC

The proportions of alkenylether moieties in PE and PC separated from culled chicken skin were 31 and 5 mol%, respectively (Table 2). This indicates that the proportions of plasmalogens were 62 mol% of PE and 10 mol% of PC, respectively. It was shown that chicken skin contained plasmenylethanolamine in high proportion. On the other hand, alkenylether composition of PS was 1 mol%.

Subsequently, PE and PC were hydrolyzed by acetolysis, and then the reaction products were developed on TLC. At least two spots were detected at Rf 0.28 and 0.12, and they were judged to be 1,2-diacyl, 3-acetyl type (from diacylphospholipid) and 1,3-diacetyl, 2-acyl type (from plasmenylphospholipid), respectively. Component fatty acids of diacyl-PE were mainly 18:0 and 20:4, while those of plasmenylethanolamine were 20:4 and 18:1 with significant amounts of HUFAs, such as docosapentanoic acid (22:5n-3) and 22:6 (Table 2). Component fatty acids of diacyl-PC were mainly 16:0 and 18:1, similar to those of the plasmenyl type. The proportion of HUFAs in plasmenylcholine was significantly less than that of plasmenylethanolamine. The fatty acids of diacyl and plasmenyl types were significantly different from fatty acids in PE and PC. Moreover, 18:0 and 16:0 were major moieties of the alkenylether group. The alkenyl group of PC was predominantly 16:0, corresponding to the result of a previous report on chicken breast [28].

Chemical Composition of Sphingolipids

Compositions of fatty acids and sphingoid bases of SM and CE separated from chicken skin are shown in Table 3. The SM consisted only of nine non-hydroxy fatty acids, with 16:0 (33.8 mol%) and 18:0 (15.7 mol%) predominating. Fatty acids with a chain as long as C24 were also present at 23.6 mol%. The CE consisted of nine non-hydroxy and six 2-hydroxy fatty acids, the former one being predominant.

Table 2 Compositions of diacyl and plasmenyl phospholipid classes (mol%)

Moieties	PE[a]		PC[b]	
	Diacyl	Plasmenyl	Diacyl	Plasmenyl
Acyl				
16:0	8.2 ± 0.2	1.8 ± 0.2	33.0 ± 1.0	38.6 ± 1.6
18:0	35.0 ± 1.1	1.3 ± 0.6	19.0 ± 0.8	15.4 ± 0.8
18:1(n-9)	14.5 ± 0.4	22.1 ± 0.1	20.2 ± 1.8	20.3 ± 0.7
18:1(n-7)	2.6 ± 0.2	–	3.5 ± 0.4	2.2 ± 0.5
18:2	11.0 ± 0.5	5.9 ± 0.4	16.7 ± 0.3	9.9 ± 0.2
20:4(n-6)	24.9 ± 1.3	47.6 ± 0.8	6.2 ± 0.5	10.5 ± 0.8
22:3(n-6)	0.4 ± 0.1	3.6 ± 1.0	0.2 ± <0.1	0.5 ± 0.1
22:5(n-3)	1.3 ± 0.1	6.5 ± 0.8	0.6 ± 0.1	0.9 ± 0.3
22:6(n-3)	2.1 ± 0.1	11.2 ± 0.4	0.6 ± 0.1	1.7 ± 0.1
Alkenylether (dimethylacetal)				
16:0		39.6 ± 1.3		82.3 ± 1.0
18:0		48.6 ± 2.2		11.8 ± 0.2
18:1(n-9)		9.8 ± 0.3		4.5 ± 0.5
18:1(n-7)		1.2 ± 0.8		1.4 ± 0.3
20:0		0.8 ± 0.2		–
Acyl:Alkenyl		69:31		95:5

Values are the means of more than duplicate analyses

[a] PE phosphatidylethanolamine

[b] PC phosphatidylcholine

Table 3 Compositions of sphingolipid classes in chicken skin (mol%)

Constituents	SM[a]	CE[b]
Fatty acid		
16:0	34.5	9.7
18:0	16.1	23.2
18:1	0.1	10.2
20:0	8	2.2
22:0	13.4	10.1
23:0	3.8	4.5
24:0	10.4	12.4
24:1(n-9)	12.5	9.5
24:2(n-6)	1.2	0.7
18h:0	–	1.5
20h:0	–	0.7
22h:0	–	5.2
23h:0	–	2.4
24h:0	–	6.2
24h:1(n-9)	–	1.5
Sphingoid base		
d18:0	0.2	6.3
d18:1^{4trans}	99.8	93.7

Values are the means of duplicate analyses

[a] SM sphingomyelin

[b] CE cerebroside

Major non-hydroxy fatty acids were 18:0 and lignoceric acid (24:0), and 2-hydroxy fatty acids were mainly 2-hydroxylignoceric (24h:0) and 2-hydroxybehenic acids (22h:0). As component sphingoid base, two dihydroxy bases, 4-*trans* sphingenine (d18:1) and sphinganine (d18:0), were detected in SM and CE, the former commonly being predominant.

This study revealed that the component sphingoid base in chicken skin sphingolipids was almost totally d18:1 similar to that of swine and human epidermis [29]. Currently, plant cerebrosides are on market as sphingolipid supplement for skin care. However, the plant origin sphingoid bases are hardly absorbed different from animal sphingoid base [16, 17]. Chicken skin SM with mainly sphingosine is considered to be human-type sphingolipid, which can exert high bioavailability.

Preparation of Concentrates of Complex Lipids from Chicken Skin

Silica gel TLC of each fraction shown in Fig. 1 and the total lipids extracted by Folch's method is shown in Fig. 4. The total lipids (lane 1) predominantly consisted of TAG and minimal levels of complex lipids. In contrast, complex lipid components could be clearly found in sample A (lane 2), which was extracted by ethanol from defatted skin, although a high amount of TAG remained. Moreover, complex lipids in chicken skin were found to be exclusively concentrated in sample B (lane 3). Meanwhile, this fraction also contained a small amount of cholesterol. Usually, it is considered that solvent fractionation of extracts from animal skins causes the contaminant of much cholesterol into the complex lipid fraction due to its high amount in animal skins; however, this solvent fractionation was effective for chicken skin with a small amount of cholesterol.

The yields of samples A (lane 2) and B (lane 3) were 14 and 2.5 g/100 g skin, respectively, while the total lipid content was 32 g/100 g skin as described above. The yield of complex lipid concentrates (sample B) corresponded closely to that (2 wt%) of complex lipids prepared from the total lipids by silicic acid column chromatography. Moreover, when yields of phospholipids obtained by this method were determined by quantifying inorganic phosphorus in each fraction, ethanol extract (sample A) and complex lipid concentrates (sample B) were 98 and 92 wt%, respectively, per total phosphorus amount in the total lipids. Thus, it was shown that complex lipids in chicken skin could be easily concentrated by this solvent fractionation without any loss. In addition, a sphingolipid-rich fraction (lane 4) was prepared by alkaline and acidic treatments of the ethanol extract (sample A), as shown in Fig. 2. These fractions extracted by ethanol contained highly polar and/or hydrophilic non-lipid components, since they could be extracted

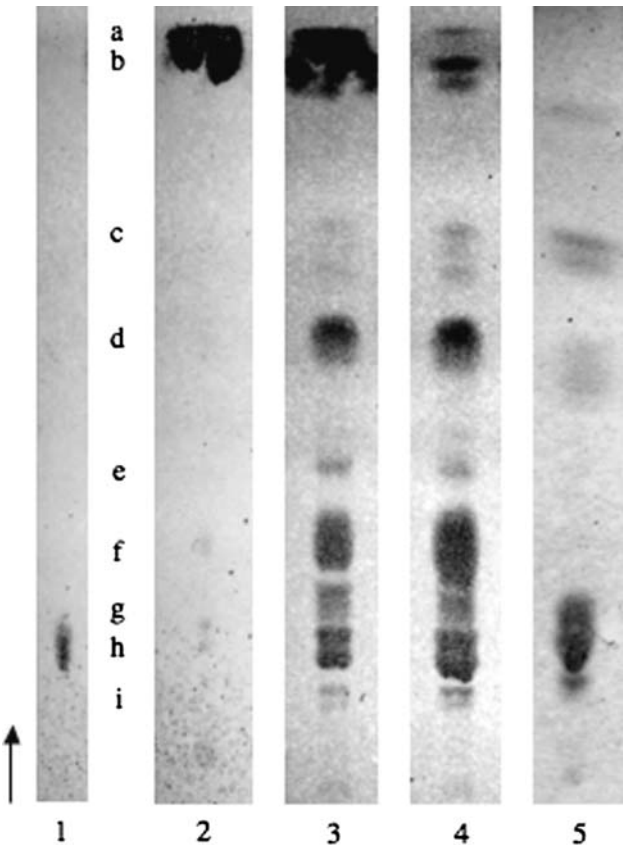

Fig. 4 TLC of neutral and complex lipids in chicken skin. *Lane 1* is standard sphingomyelin. *Lane 2* is total lipids obtained by Folch's method. *Lanes 3* and *4* correspond to samples A and B in Fig. 1, respectively. *Lane 5* corresponds to sphingomyelin in Fig. 2. TLC plate was developed with chloroform–methanol–water (65:25:4 v/v/v) in the direction of the *arrow* shown in figure. Spots *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h* and *i* indicate triacylglycerol, cholesterol, cerebroside, phosphatidylethanolamine, lactosylceramide, phosphatidylcholine, sphingomyelin, phosphatidylserine and lysophosphatidylcholine, respectively. Detection was carried out by spraying 50% H_2SO_4 followed by a brief heating at 200 °C

with ethanol. These can be almost totally removed by washing with 50% aqueous acetone and water. As a result, the SM concentrate (lane 4) was found to be 95% pure by HPLC analysis.

This study demonstrated that culled chicken skin could be a readily available source of functional complex lipids such as SM and plasmalogens, although it was only on a laboratory scale. An investigation of large-scale production to follow up this laboratory scale experiment is necessary to determine the feasibility of using culled chicken skin as a source of functional lipids.

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