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CHROMATOGRAPHIC RESOLUTION OF MOLECULAR SPECIES OF PHOSPHATIDYLSERINES AND PHOSPHATIDYLETHANOLAMINES AS THEIR N-TRIFLUOROACETYL-O-METHYL AND N-ACETYL-O-METHYL DERIVATIVES

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SUMMARY

A new technique is described for preparing the N-trifluoroacetyl-O-methyl derivatives of phosphatidylserine and phosphatidylethanolamine. It is rapid, and the derivatives are well separated into the following main molecular species by argentation thin-layer chromatography: saturated-monoene, saturated-dienoic, monoene-dienoic, diene-dienoic, saturated-trienoic, saturated-tetraenoic, saturated-pentaenoic and saturated-hexaenoic. The procedure gave full recovery of phosphatidylethanolamine, while phosphatidylserine in addition gave varying amounts of another derivative, probably a cyclic azlactone. The N-acetyl-O-methyl derivatives were therefore also prepared by a similar procedure. These derivatives separated similarly well on argentation chromatography, and both phosphatidylserine and phosphatidylethanolamine gave full recoveries. The two techniques have been used to analyze rat liver phosphatidylserine and phosphatidylethanolamine.

INTRODUCTION

Several systems for the chromatographic separation of intact phospholipids into subclasses according to fatty acid composition have been described for lecithins [1], phosphatidylethanolamines [1—4], phosphatidylinositols [5] and phosphatidylserines [3, 5]. The best resolution has in general been obtained when the phospholipids were converted to apolar diacylacetyl- or diacylglycerols [7, 8], or dimethylphosphatidates [9]. Part of the molecule is thereby lost, which is a disadvantage in biological studies. However, blocking of the polar groups of the phospholipids can give comparable resolution [2].

Separation of intact phosphatidylserine gives a very poor separation [6]. The N-dinitrobenzamide-O-methyl derivative gave a good resolution, but gave

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low recoveries [10]. The preparation of the trifluoroacetamides gave a relatively good separation, although the carboxyl and hydroxyl groups were not derivatized [3]. However, the authors did not state the recovery of their procedure, and also stated that varying amounts of a azlactone derivative was formed together with the trifluoroacetamides [3]. In our hands, this procedure gave very low recoveries using small amounts of phosphatidylserine, and we therefore investigated the possibility of preparing N-acyl-O-methyl derivatives with high recovery.

MATERIALS AND METHODS

Preparation of rat liver phosphatidylethanolamine and phosphatidylserine

Male Wistar rats weighing 180—250 g were decapitated, the livers rapidly removed, weighed and homogenized in 30 ml of ice-cold methanol containing 1.5 mg of 2.6-di-*tert.*-butyl-*p*-cresol (BHT) as antioxidant. Lipids were extracted from this homogenate according to the method of Bligh and Dyer [11]. Phosphatidylethanolamine and phosphatidylserine were isolated by thin-layer chromatography (TLC) on 0.5 mm silica gel H (Merck, Darmstadt, G.F.R.) layers containing Na_2CO_3 [12], using chloroform—methanol—acetic acid—water (50:25:7:3) as developing solvent. The fractions were located by spraying with 0.02% dichlorofluorescein in ethanol, and eluted [1]. The phosphatidylserine fraction was further purified by TLC on Na_2CO_3-impregnated silica gel H using chloroform—methanol—acetic acid—water (50:15:12:6) as solvent. The purity was finally checked by TLC on silica gel H using chloroform—methanol—conc. ammonia—water (60 : 35 : 2.5 : 2.5) as solvent, and was better than 98% calculated from phosphorus content. The phospholipids were stored at −20°C under nitrogen in chloroform containing 50 mg/l BHT.

Preparation of N-trifluoroacetyl-O-methyl derivatives

Phosphatidylserine or phosphatidylethanolamine (0.03—1.5 μmoles) was dried under nitrogen in a 5-ml reagent tube, and dissolved in 1 ml of freshly distilled chloroform containing 20 μl of trifluoroacetic anhydride (Fluka, Buchs, Switzerland). The tube was flushed with nitrogen and closed with a Teflon-lined stopper. After 5 min at room temperature, excess trifluoroacetic anhydride was removed by dropwise addition of a 0.2—0.3 mol/l solution of freshly prepared diazomethane in diethyl ether [13]. Diazomethane was added in slight excess as indicated by a persisting light yellow colour of the reaction mixture. After 20 min at room temperature the reaction mixture was taken to dryness under nitrogen and dissolved in a small volume of chloroform. The derivatives were isolated by TLC on silica gel H using chloroform—isopropanol (96 : 5) as solvent, and located by spraying with 0.02% dichlorofluorescein in ethanol before elution from the gel [1].

Preparation of N-acetyl-O-methyl derivatives

Phosphatidylserine or phosphatidylethanolamine (0.03—1.5 μmoles) was dried under N_2 and dissolved in 1 ml of freshly distilled chloroform containing 25 μl of acetic anhydride. After 30 min at room temperature, 1.5 ml of freshly distilled diethyl ether was added, and then freshly prepared 0.2—0.3 mol/l

diazomethane in diethyl ether [13] was added dropwise in slight excess as indicated by a persisting light yellow colour. After 20 min at room temperature, the reaction mixture was evaporated at 40°C under nitrogen until all traces of acetic anhydride had been removed. The derivatives were isolated by TLC as described for the N-trifluoroacetyl-O-methyl derivatives.

Argentation chromatography

Thin-layer plates (20 × 20 cm, 0.5 mm thickness) containing 12 g of silver nitrate per 40 g of silica gel H were activated at 180°C for 2 h, and stored over phosphorus pentoxide until used. The solvent systems are described in the figures. Fractions were visualized after spraying with 0.002% dichlorofluorescein in ethanol, viewed under ultraviolet light, and eluted [1] and washed with 0.45% sodium chloride in water—methanol (1:1). Aliquots were taken for phosphorus and fatty acid analysis.

Other methods

The relative abundance of fatty acids in the lipid fractions was determined after transmethylation with 14% boron trifluoride in methanol at 90°C for 10 min in a nitrogen atmosphere [14]. The methyl esters were chromatographed on a SP-2330 capillary column (30 m × 0.25 mm I.D.) using a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector, an injector split ratio of 1:30, and argon as carrier gas. Column temperature was 195°C, injector and detector temperatures were both 250°C. The fatty acids were identified by comparing retention times with authentic standards. Phosphorus was determined according to the method of Bartlett [15], and liquid scintillation counting was performed in a Packard TriCarb 3385 instrument using Insta gel II (Packard) as the scintillation fluid.

RESULTS AND DISCUSSION

The efficacy of the two different derivatisation procedures was checked both by phosphorus analysis of the derivatives and by preparing ^{14}C-labeled phosphatidylserines and -ethanolamines isolated from rat liver cells incubated with L-[^{14}C]serine [16]. N-Trifluoroacetyl-O-methyl-phosphatidylserines and -ethanolamines were both rapidly formed, and the derivatives separated well on silica gel H using chloroform—isopropanol (96:5) as solvent (R_F values 0.45 and 0.30, respectively). The N-acetyl derivatives formed more slowly, and had R_F values of 0.55 and 0.40, respectively. No traces of dimethylphosphatidates [17] could be detected using the present, low concentrations of diazomethane. Phosphatidylinositols did not produce any interfering derivatives during these procedures, as all material remained at the origin.

Recovery of the N-trifluoroacetyl-O-methyl-phosphatidylethanolamines was 90—98% based on phosphorus, while recovery of the corresponding N-acetyl derivatives was 99—100% based on phosphorus and 93—97% based on radioactivity. Recovery of the N-acetyl-O-methyl-phosphatidylserines was 93—102% based on phosphorus and 95—98% based on radioactivity, while the corresponding N-trifluoroacetyl derivatives gave a varying recovery from 45 to 93% by both phosphorus and radioactivity analysis. The remainder was found in

TABLE I

MOLAR FATTY ACID COMPOSITION OF RAT LIVER PHOSPHATIDYLSERINES AND PHOSPHATIDYLETHANOLAMINES AND THEIR N-TRIFLUOROACETYL-O-METHYL AND N-ACETYL-O-METHYL DERIVATIVES

In each experiment, phospholipids from one rat liver were isolated and derivatized as described in Methods.

	Moles per cent							
	Experiment I		Experiment II		Experiment I		Experiment II	
	Original PS*	N-TFA-O-Me-PS*	Original PS	N-Ac-O-Me-PS*	Original PE*	N-TFA-O-Me-PE*	Original PE	N-Ac-O-Me-PE*
16:0	3.1	3.1	4.3	3.0	21.5	21.2	16.7	18.1
16:1	0.1	0.3	0.1	0.2	0.3	0.3	0.5	0.5
18:0	46.6	47.3	47.0	50.2	24.4	24.3	28.1	27.8
18:1	2.2	2.3	3.3	3.7	5.0	4.8	6.0	6.3
18:2	3.6	3.4	3.2	2.7	7.0	7.1	6.4	6.4
18:3	—	—	—	—	0.5	0.4	0.2	0.2
20:1	—	—	—	—	0.3	0.3	—	—
20:2**	—	—	—	—	0.3	0.3	0.2	0.2
20:3	1.3	1.0	0.7	0.8	0.6	0.6	0.7	0.6
20:4	21.9	20.2	17.5	16.8	19.5	19.3	19.1	18.0
20:5	0.7	0.6	1.4	0.9	1.1	1.4	1.7	1.7
22:4**	—	1.0	0.4	0.4	—	—	0.3	0.3
22:5	1.9	1.8	1.9	1.8	2.9	2.9	2.2	2.2
22:6	18.3	18.5	20.2	19.5	16.3	16.9	17.9	17.7

*PS = phosphatidylserine; PE = phosphatidylethanolamine; N-TFA-O-Me- = N-trifluoroacetyl-O-methyl-; N-Ac-O-Me- = N-acetyl-O-methyl-.
**Tentatively identified.

another, more polar, derivative, which was tentatively identified as the cyclic azlactone [3]. No azlactones were formed during N-acetylation.

The fatty acid composition of both the N-trifluoroacetyl and the N-acetyl derivatives of phosphatidylserines and phosphatidylethanolamines was identical to that of the intact phospholipids (Table I). The azlactones formed during trifluoroacetylation of phosphatidylserine also had the same fatty acid composition (not shown). These results indicate that no selective loss of any particular molecular phospholipid species occurred during any of the two derivatisation procedures, although the recovery of the N-trifluoroacetyl-O-methyl-phosphatidylserines was variable.

Argentation chromatography of the phospholipid derivatives is shown in Figs. 1 and 2. The rat liver phosphatidylserines were clearly separated into the following fractions: saturated-hexanoic, saturated-pentanoic, saturated-tetraenoic, saturated-trienoic, diene-dienoic, monoene-dienoic, saturated-dienoic and saturated-monoenic. The saturated-trienoic fraction was very low in rat liver, and was therefore included in the tetraenoic fraction. Similarly, the diene-dienoic fraction from rat liver was combined with the monoene-dienoic fraction. The N-trifluoroacetyl-O-methyl-phosphatidylethanolamines separated into similar fractions as previously reported for the corresponding N-acetyl-O-

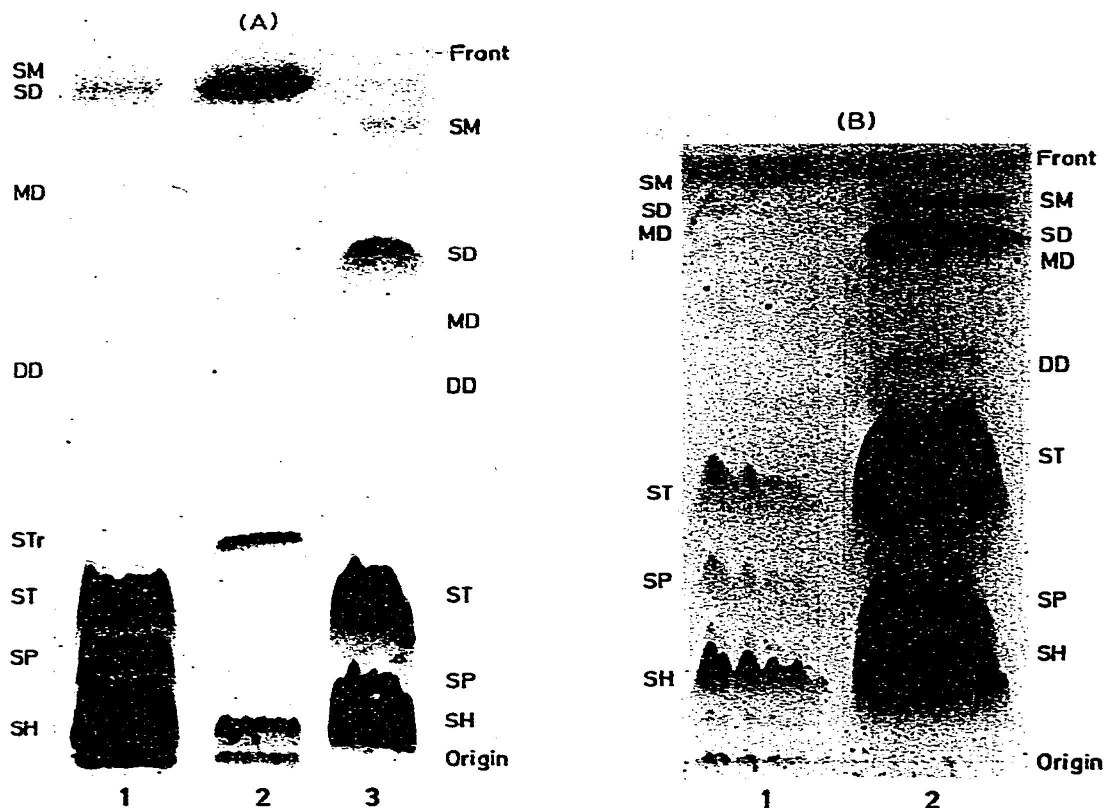

Fig. 1. Separation of N-trifluoroacetyl-O-methyl derivatives of phosphatidylserine and phosphatidylethanolamine. (A) 1 = Rat liver phosphatidylserine, 0.3 μmole; 2 = ox brain phosphatidylserine (Sigma, St. Louis, MO, U.S.A.), 0.4 μmole; 3 = rat liver phosphatidylethanolamine, 0.4 μmole. The plate was first developed for 10 cm with chloroform—methanol—water (80:15:2), briefly dried, and then developed to 1 cm from the top with chloroform—methanol (97:3). (B) 1 = Rat liver phosphatidylserines, 0.3 μmole; 2 = rat liver phosphatidylethanolamines, 1.2 μmole. The first solvent (chloroform—methanol—water, 80:20:3) was run for 10 cm, the plate briefly dried and then developed in chloroform—methanol (99:1). Spots were visualized by charring after spraying with 48% sulfuric acid. The fatty acid composition of the fractions is indicated along the side of the chromatograms. Abbreviations: S = saturated, M = monoene, D = diene, Tr = triene, T = tetraene, P = pentaene, H = hexaene.

methyl derivative [2]. The N-acyl-O-methyl derivatives thus seem to give a better chromatographic resolution than reported for the trifluoroacetamides of phosphatidylserine and -ethanolamine, or for the azlactone of phosphatidylserines [3].

The fatty acid composition of isolated subfractions of N-acetyl-O-methylphosphatidylserines and N-trifluoroacetyl-O-methyl-phosphatidylethanolamines is shown in Tables II and III. The phosphatidylethanolamine fractions were similar to that reported previously [2]. The saturated-hexaenoic, saturated-tetraenoic and saturated-dienoic fractions of rat liver phosphatidylserines seem to be nearly unimolecular, with stearic acid as the dominating saturated fatty acid. The monoene-dienoic fraction is the most inhomogeneous fraction and

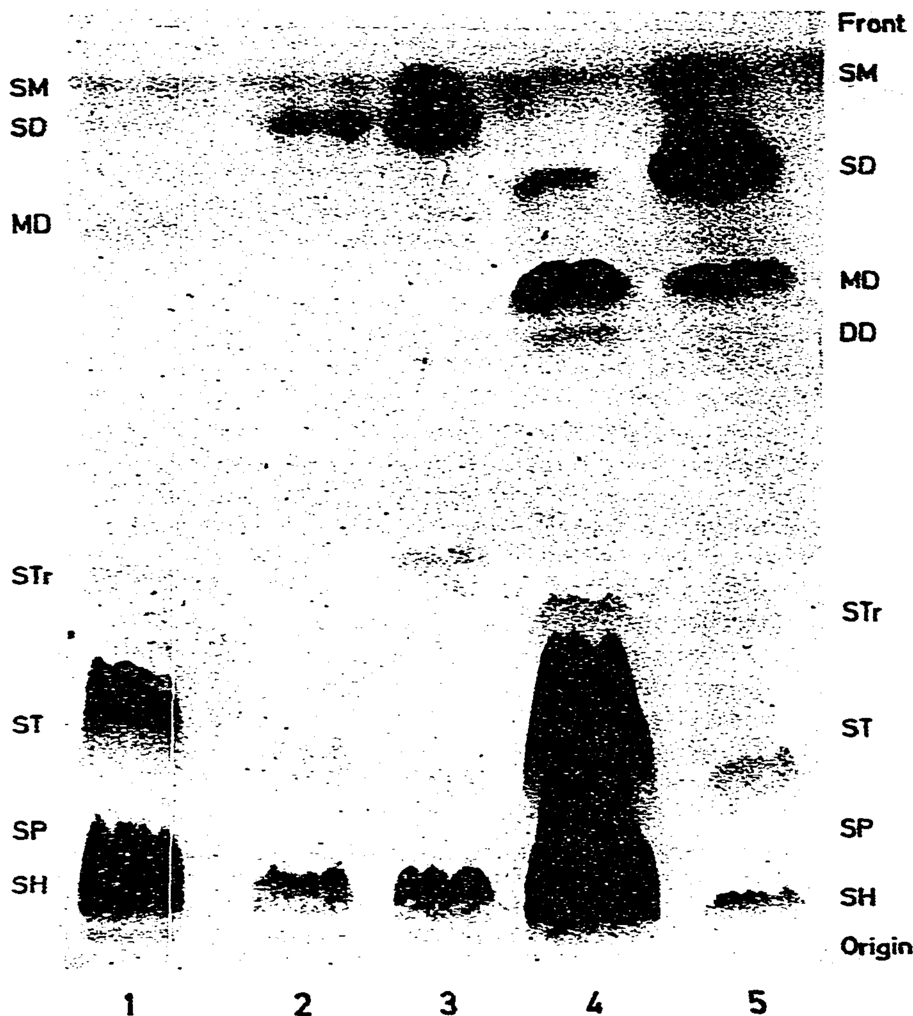

Fig. 2. Separation of N-acetyl-O-methyl derivatives of phosphatidylserines and phosphatidylethanolamines. 1 = Rat liver phosphatidylserines, 0.1 μmole; 2 = ox brain phosphatidylserines (Sigma), 0.05 μmole; 3 = ox brain phosphatidylserines (Koch-Light, Colnbrook, Great Britain), 0.1 μmole; 4 = rat liver phosphatidylethanolamines, 0.3 μmole; 5 = egg phosphatidylethanolamines, 0.1 μmole. The first solvent was chloroform—methanol—water (80:20:3) which was run for 10 cm. After a brief drying, the plate was run to 1 cm from the top with chloroform— methanol (97:3). Abbreviations and visualization as in Fig. 1.

could be seen to consist of several small fractions on the $AgNO_3$ plates. The saturated-monoene fraction seems to contain some disaturated species, and a rather high concentration of lignoceric acid was found in this fraction. For both the N-trifluoroacetyl-O-methyl-phosphatidylethanolamines and the N-acetyl-O-methyl-phosphatidylserines, summing the individual fatty acids according to the relative abundance of each fraction gives a calculated fatty acid composition of the total phosphatidylserines and phosphatidylethanolamines which agrees closely with that found in the intact phospholipids.

The present work is the first report on argentation chromatography of

TABLE II

FATTY ACID COMPOSITION OF N-ACETYL-O-METHYL-PHOSPHATIDYLSERINE MOLECULAR SPECIES ISOLATED FROM RAT LIVER

Abbreviations as in Fig. 1.

	Fatty acid composition (moles per cent)							
	Fraction (relative amount)						Total PS measured	Total PS calculated*
	SH (48.5%)	SP (6.3%)	ST (35.7%)	MD (1.0%)	SD (4.6%)	SM (3.9%)		
14:0	0.2	0.8	0.2	2.6	0.8	2.0	0.2	0.3
14:1	0.8	0.3	1.3	0.4	0.2	0.3	0.5	0.9
16:0	2.6	5.2	2.9	16.0	6.3	7.3	2.9	3.3
16:1	0.1	0.3	0.1	4.3	0.2	4.3	0.2	0.3
16:2**	1.2	1.1	1.2	2.7	1.1	1.0	1.4	1.2
18:0	48.4	45.2	49.0	43.6	45.2	32.3	48.9	47.7
18:1	1.3	4.2	0.8	12.0	2.9	34.6	3.1	2.8
18:2	0.1	0.7	0.4	5.6	42.8	2.3	2.5	2.4
20:0	—	—	—	—	—	1.0	0.2	—
20:1	—	—	—	—	—	1.0	—	—
20:2***	—	—	—	9.5	—	—	0.2	0.1
20:3	0.1	—	1.9	0.7	—	—	0.7	0.7
20:4	1.9	12.0	41.3	0.6	—	—	16.4	16.4
20:5	0.7	9.6	—	—	—	—	0.9	0.9
22:0	—	—	—	—	—	1.4	—	0.1
22:1	—	—	—	—	—	0.2	—	—
22:4***	—	0.9	0.9	—	—	—	0.3	0.4
22:5	1.5	18.1	—	—	—	—	1.8	1.9
22:6	41.1	1.2	—	—	—	—	19.4	20.0
24:0	—	0.4	—	2.0	0.5	12.3	0.4	0.6

*Calculated from the relative amount of each subfraction and its fatty acid composition.
**Includes dimethylacetals.
***Tentatively identified.

N-trifluoroacetyl-O-methyl and N-acetyl-O-methyl derivatives of phosphatidylserines. It also for the first time describes the separation of N-trifluoroacetyl-O-methyl-phosphatidylethanolamines. The O-methylation gives better chromatographic resolution compared to trifluoroacetylation only. Further, the present method is considerably more rapid than those published previously [2, 3]. The most important point, however, is that the present procedure for preparing N-acetyl-O-methyl-phosphatidylserines gives a complete recovery of the derivatives, also when using very small amounts of the phospholipid.

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TABLE III

FATTY ACID COMPOSITION OF N-ACETYL-O-METHYL-PHOSPHATIDYLETHANOLAMINE MOLECULAR SPECIES ISOLATED FROM RAT LIVER

Abbreviations as in Fig. 1.

	Fatty acid composition (moles per cent)								
	Fraction (relative amount)							Total PE measured	Total PE calculated*
	SH (24.0%)	SP (7.7%)	ST (45.8%)	DD (0.7%)	MD (4.6%)	SD (14.2%)	SM (3.1%)		
14:0	0.2	—	—	1.4	0.3	0.3	—	0.3	0.1
14:1	0.5	—	—	0.2	—	—	—	0.1	0.1
16:0	33.4	21.8	12.2	13.9	9.2	25.8	25.9	22.6	19.7
16:1	0.9	1.1	0.3	3.0	1.8	0.6	—	0.4	0.6
18:0	11.6	15.0	28.5	18.2	2.5	20.6	10.3	23.0	20.5
18:1	5.1	6.8	6.3	9.9	37.0	4.8	33.6	9.0	8.1
18:2	0.7	4.5	1.9	24.5	49.2	46.2	—	9.9	10.4
18:3	—	—	0.5	5.9	—	0.8	—	0.4	0.4
20:1	—	—	—	6.3	—	1.1	30.2	0.2	1.0
20:2**	—	—	—	16.9	—	—	—	0.4	0.1
20:3	0.8	3.2	1.6	—	—	—	—	0.8	1.2
20:4	0.6	11.6	47.7	—	—	—	—	20.0	22.9
20:5	1.0	11.9	0.2	—	—	—	—	1.4	1.3
22:3**	—	0.2	0.6	—	—	—	—	0.2	0.3
22:4**	—	0.4	—	—	—	—	—	—	—
22:5	2.4	23.2	0.1	—	—	—	—	2.4	2.4
22:6	43.5	0.4	0.1	—	—	—	—	8.9	10.5

*Calculated from the relative amount of each fraction and its fatty acid composition.
**Tentatively identified.

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