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Identification and quantification of molecular species of diacyl glyceryl ether by reversed-phase high-performance liquid chromatography with refractive index detection and mass spectrometry

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Abstract

We have developed a method to identify and quantify the molecular species of diacyl glyceryl ether (DAGE) using high-performance liquid chromatography (HPLC) equipped with a refractive index detector and an electrospray ionization and time of flight mass spectrometer (LC–RI–MS). An octadecyl silica column with a mixture of acetonitrile and dichloromethane (65:35, v/v) as an eluant was used for the HPLC. When the LC–RI–MS method was applied to a mixture of synthetic DAGEs; 1-*O*-hexadecyl-2,3-dioleoylglycerol (*O*-16:0–18:1–18:1), 1-*O*-octadecyl-2,3-dioleoylglycerol (*O*-18:0–18:1–18:1), 1-*O*-octadecenyl-2,3-dioleoylglycerol (*O*-18:1–18:1–18:1), 1-*O*-octadecyl-2,3-didocodahexaenoylglycerol (*O*-18:0–22:6–22:6), and 1-*O*-octadecenyl-2,3-didocosahexaenoylglycerol (*O*-18:1–22:6–22:6), good separation and quantification were obtained on the refractive index chromatogram. A pseudo-molecular ion $[M+NH_4]^+$ and a monoacyl glyceryl ether ion $[M-RCO_2]^+$ were observed for all synthetic DAGEs on the mass spectrum. It was found that the fatty acids and glyceryl ether in DAGE could be easily identified by these mass spectra. When this LC–RI–MS method was applied to the DAGEs extracted from muscle of *Stromateus stellatus*, approximately 18 peaks were observed on LC–RI–MS chromatograms and the major molecular species of DAGEs were identified as *O*-16:0–18:1–18:1.

Keywords: Refractive index detection; Diacyl glyceryl ether; Fatty acids

1. Introduction

Most marine animals, such as fish, whales and seals, have lipids that are rich in triacylglycerol (TAG) in the body. However, some marine animals contain diacyl glyceryl ethers (DAGEs) as the major lipids in the body. Deep-sea teleost fish, deep-sea shark and gonatid squid have considerable amounts of DAGEs in the muscle and liver [1–5]. The physiological function and importance of DAGE in marine animals have not been clarified yet, although Malins and Barone [6] suggested that DAGE might attribute to buoyancy in deep-sea fishes such as dogfish. General TAGs are edible, whereas DAGEs may cause food-poisoning, leading to diarrhea and

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an upset stomach. It is thought that the toxicity of DAGE is due to poor digestion and absorption, due to the ether bond's resistant to hydrolysis by pancreatic lipases [1]. Accordingly, the presence of DAGE is occasionally investigated for marine foods from the food-hygiene standpoint. In particular, the fatty acid and glyceryl ether compositions of DAGEs are conventionally measured by gas liquid chromatography (GLC). Iida [2] and Mori et al. [3] found that unsaponifiables made up approximately 30% of the total lipids in the muscle of three deep-sea teleost fish (*Cubiceps gracilis*, *Centrolophus* sp. and *Stromateus maculatus*), and that most of them were glyceryl ether. Major glyceryl ethers are chimyl alcohol (1-*O*-hexadecylglycerol) and selachyl alcohol (1-*O*-octadecenylglycerol), while major fatty acids in DAGEs are C16:0 and C18:1.

Hayashi and Takagi [4] found that DAGEs were present in the muscle, liver and ovaries of the deep-sea shark (*Dalatias licha*), and that DAGEs were extremely abundant in the ovary. They also reported that DAGEs mainly consisted of monounsaturated fatty acids, such as C18:1, C20:1 and C22:1, and selachyl alcohol, as a glyceryl ether, in all of the tissues of the deep-sea shark. Hayashi and Kawasaki [5] investigated the fatty acid and glyceryl ether composition of DAGEs that were abundant in the liver of two gonatid squids (*Berryteuthis magister* and *Gonatopsis makko*). Polyunsaturated fatty acids, such as C20:5 and C22:6, were present in DAGEs extracted from the liver of the gonatid squid, in addition to C18:1. However, these data was obtained only by GLC, and the molecular species of those DAGEs have not been clarified yet, since an analytical method to determine the molecular structure of DAGEs has not been established. The presence of many types of fatty acids and glyceryl ether and the absence of commercial DAGEs made the analysis of the molecular species of DAGE difficult. However, it is important to know the composition of DAGEs' molecular species, because toxic activity may depend on the molecular structure of the DAGE. The analysis of DAGE molecular species may also be useful for the characterization of fish species.

High-temperature GLC [7] and high-performance liquid chromatography (HPLC) with refractive index (RI) detection [8,9], evaporative light scattering detection [10,11] and mass spectrometry (MS) are often used as a method to characterize molecular species of TAG contained in vegetable oil and fish oil. In particular, MS provides fast atom bombardment (FAB) [12,13], electrospray ionization (ESI) [14] and atmospheric pressure chemical ionization (APCI) methods [15–19], which can be used to determine the molecular mass of the TAG. In this paper, we describe a method we have developed to identify and quantify the molecular species of natural DAGEs using a HPLC system equipped with an RI detector and an electrospray ionization and time of flight (ESI/TOF)–MS (LC–RI–MS).

2. Experimental

2.1. DAGEs

1-*O*-Hexadecyl-2,3-dioleoylglycerol (*O*-16:0–18:1–18:1), 1-*O*-octadecyl-2,3-dioleoylglycerol (*O*-18:0–18:1–18:1), 1-*O*-octadecenyl-2,3-dioleoylglycerol (*O*-18:1–18:1–18:1), 1-*O*-octadecyl-2,3-didocosahexaenoylglycerol (*O*-18:0–22:6–22:6) and 1 - *O* - octadecenyl - 2, 3 - didocosahexaenoylglycerol (*O*-18:1–22:6–22:6) were prepared by chemical esterification of oleic acid (C18:1) and docosahexaenoic acid (C22:6) to 1-*O*-hexadecylglycerol, 1-*O*-octadecylglycerol or 1-*O*-octadecenylglycerol (Sigma, St. Louis, MO, USA), according to the method described in a previous paper [20]. These synthetic DAGEs are racemic.

Deep-sea teleost fish (*Stromateus stellatus*), which was a source of food poisoning in Osaka and Nagano Prefectures in Japan in 1999, was provided from the Osaka Prefectural Government, Medical Administration Division, Public Health Department. Lipids were extracted from the fish muscle using the Bligh–Dyer method [21] and were then loaded on a Florisil (deactivated with water by 5%) column to isolate DAGEs from TAGs whose peaks might overlap with some peaks of DAGE species on a HPLC chromatogram. DAGE was eluted with diethyl ether and *n*-hexane (5:95, v/v) and concentrated using a rotary evaporator.

All DAGEs were stored at −30°C prior to LC–RI–MS analysis, because they were susceptible to oxidation.

2.2. LC–RI–MS analytical system

For the LC–RI–MS analytical system, the HPLC column was a 250×4.6 mm, 5 μm Inertsil ODS-2 (Showa Denko Co., Japan). The mobile phase was a mixture of dichloromethane and acetonitrile (35:65, v/v) and the flow-rate was 1.0 ml/min. The column temperature was 35°C. After the column eluant passed through the Shodex RI-71 refractive index detector (Showa Denko Co.) at 35°C, it was mixed with a mixture of chloroform and methanol (1:1, v/v) containing 0.2 m*M* ammonium acetate at 1.0 ml/min in a post column-mixing joint. One of 20 volumes of eluant was passed through a Mariner ESI/TOF–MS (PerSeptive Biosystems, France). MS spectrometry was performed with the positive-ion measurement mode with a spray voltage of 4000 V, a nozzle potential of 150 V, and a nozzle temperature of 150°C. The flow-rate of nebulizer gas was 0.3 ml/min. Full scan spectra were obtained by scanning masses between *m/z* 300 and 1100 at 4 s/scan.

DAGEs (500–600 mg) were precisely weighed and carefully dissolved in dichloromethane in a volumetric flask. A 10-μl volume of the solution (500 μg–12 mg/ml) was injected into the column using a Rheodyne Model 7715 loop (20 μl) injector.

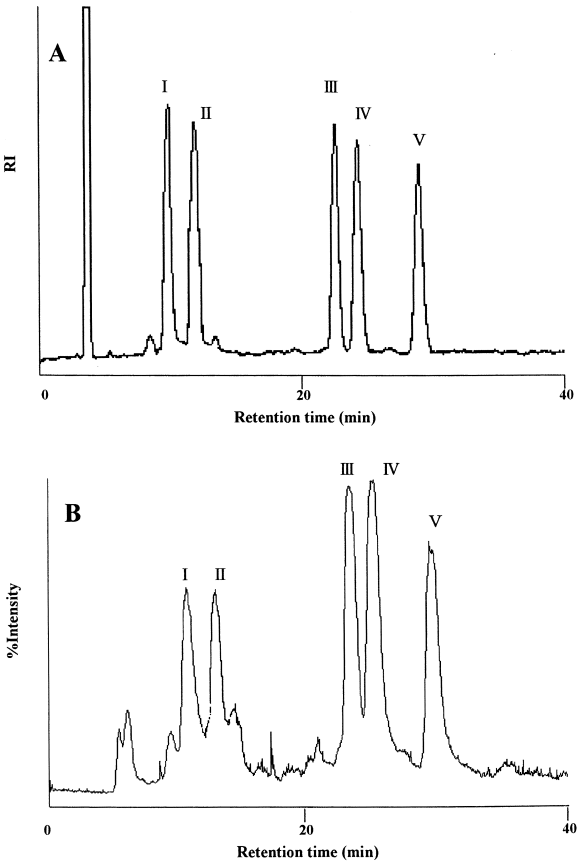

Fig. 1. Refractive index (A) and total ion (B) chromatograms of synthetic diacylglyceryl ethers in LC–RI–MS. For chromatographic conditions, see Experimental. I, 1-*O*-octadecenyl-2,3-didocosahexaenoylglycerol; II, 1-*O*-octadecyl-2,3-didocosahexaenoylglycerol; III, 1-*O*-octadecenyl-2,3-dioleoylglycerol; IV, 1-*O*-hexadecyl-2,3-dioleoylglycerol, and V, 1-*O*-octadecyl-2,3-dioleoylglycerol.

3. Results and discussion

3.1. LC–RI–MS separation

Initially, a mixture containing 100 μg of synthetic DAGEs was injected into the LC–RI–MS system. Fig. 1 shows chromatograms of synthetic DAGEs obtained by calculation of the RI- and the total ion current number under optimal conditions, respectively. There was good separation of five types of DAGEs (*O*-16:0–18:1–18:1, *O*-18:0–18:1–18:1, *O*-18:1–18:1–18:1, *O*-18:0–22:6–22:6, and *O*-18:1–22:6–22:6) on both chromatograms when using a mixture of dichloromethane and acetonitrile as the mobile phase, although the post-RI dilution at atmospheric pressure gave rise to somewhat unavoidable peak-broadening on the chromatogram of total ions (Fig. 1B). The elution order was as follows; *O*-18:1–22:6–22:6 (10–11 min), *O*-18:0–22:6–22:6 (12–13 min), *O*-18:1–18:1–18:1 (22–23 min), *O*-16:0–18:1–18:1 (24–25 min), and *O*-18:0–18:1–18:1 (28–29 min). Retention times (Rts) of DAGEs depended on the type of fatty acid and glyceryl ether present. The conditions used for HPLC analysis were not sufficient to individually separate racemic DAGEs. However, it is not always necessary to separate racemic DAGEs because most of the naturally occurring DAGEs have an ether bond at the 1-position of the glycerol backbone but not at the 3-position [22].

3.2. Quantitative and qualitative analysis by LC–RI–MS

Standard solutions of synthetic DAGEs were injected into the LC–RI–MS system over the range of 5 to 120 μg. Fig. 2 shows the relationships between the molecular mass of DAGEs and their peak area on the RI chromatogram. A good and positive correlation (r^2=0.954) was observed, as shown in Fig. 2, and it was independent of the type of DAGE. RI detection gave high precision, with a coefficient of variation below 5% for DAGEs. Moreover, the detection limit of DAGEs on the RI chromatogram was 1 μg.

In contrast, the molecular mass of DAGEs did not always correlate with the total ion intensity in the range of concentrations used. Moreover, the total ion intensity of DAGEs was affected by the type of fatty acid present. The total ion intensity was stronger for monounsaturated fatty acid-containing DAGEs than for polyunsaturated fatty acids such as docosahexaenoic acid-containing DAGEs, as shown in Fig. 1.

Fig. 3 shows the mass spectrum of *O*-18:1–22:6–22:6 as an example of a DAGE. In this example, *O*-18:1–22:6–22:6 showed a pseudo-molecular ion $[M+NH_4]^+$ at *m/z* 981 and a monoacyl glyceryl ether ion $[M-RCO_2]^+$, resulting from the loss of fatty acid from DAGE, at *m/z* 636. In a similar manner, both a pseudo-molecular ion and a monoacyl glyceryl ether ion were observed for all synthetic DAGEs (Table 1). These fragmentation patterns were very similar to those observed in TAG [18]. It was found that the molecular species of DAGE could be identified by two specific ions, although enantiomers of DAGE could not be identified using the mass spectra.

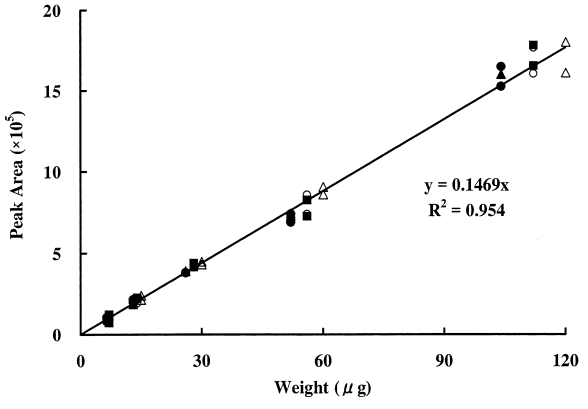

Fig. 2. Relationship between the weight and RI peak area of synthetic diacylglyceryl ethers. ○, 1-*O*-octadecenyl-2,3-didocosahexaenoylglycerol; △, 1-*O*-octadecyl-2,3-didocosahexaenoylglycerol; ●, 1-*O*-octadecenyl-2,3-dioleoylglycerol; ■, 1-*O*-hexadecyl-2,3-dioleoylglycerol, and ▲, 1-*O*-octadecyl-2,3-dioleoylglycerol.

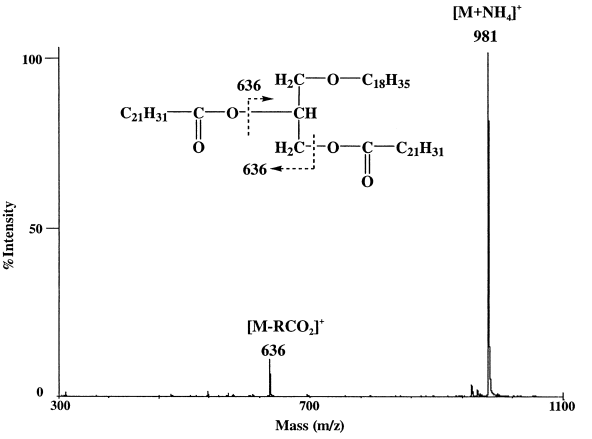

Fig. 3. Mass spectrum of 1-*O*-octadecenyl-2,3-didocosahexaenoylglycerol.

3.3. LC–RI–MS analysis of DAGEs in the deep-sea teleost fish

The LC–RI–MS method was applied to DAGEs extracted from the muscle of *Stromateus stellatus*. Fig. 4 shows the RI and total ion chromatograms of the extracted DAGEs. Approximately 18 peaks were detected at retention times ranging from 13 to 46 min on both chromatograms, and the major peak (Peak 10) was eluted at 23.7 min. Therefore, a mass spectrum was measured for each individual peak. Fig. 5 shows the mass spectra of Peaks 10 and 14. Two intensive ions due to $[M+NH_4]^+$ and $[M-RCO_2]^+$ were observed at *m/z* 863 and 564, respectively, for Peak 10. From the MS data, Peak 10 was identified as 1-*O*-hexadecyl-2,3-dioleoylglycerol (*O*-16:0–18:1–18:1). In addition, three intensive ions were found on the mass spectrum of Peak 14, as shown in Fig. 5B. These were determined to be $[M+NH_4]^+$ at *m/z* 919, and $[M-RCO_2]^+$ at *m/z* 620 and 564, respectively. Thus, Peak 14 was

Table 1
Pseudo-molecular $[M+NH_4]^+$ and monoacyl glyceryl ether $[M-RCO_2]^+$ ions of synthetic diacyl glyceryl ether (DAGE)

DAGE	MW	*m/z*	
		$[M+NH_4]^+$	$[M-RCO_2]^+$
1-*O*-Octadecenyl-2,3-didocosahexaenoylglycerol	962	981	636
1-*O*-Octadecyl-2,3-didocosahexaenoylglycerol	964	983	638
1-*O*-Octadecenyl-2,3-dioleoylglycerol	870	889	590
1-*O*-Hexadecyl-2,3-dioleoylglycerol	844	863	564
1-*O*-Octadecyl-2,3-dioleoylglycerol	872	891	592

identified as 1-*O*-hexadecyl-oleoyl-docosenoylglycerol (*O*-16:0–18:1–22:1). We also identified other peaks by MS. As shown in Table 2, at least 19 DAGE molecular species were detected.

Unfortunately, the location of the fatty acid in DAGE could not be determined based on the MS spectrum, although DAGEs contained in marine lipids usually consisted of 1-*O*-alkylglycerol [22].

Moreover, we estimated the composition of DAGE molecular species based on their peak area on the RI chromatogram. The major component was *O*-16:0–18:1–18:1, which accounted for 23% of total DAGEs. Considerable amounts of the DAGEs *O*-14:0–18:1–18:1 (11%) and *O*-16:0–18:1–22:1 (11%) were also found in *Stromateus stellatus*.

GLC analysis showed that DAGEs extracted from *Stromateus stellatus* consisted of 17 fatty acids and 10 glyceryl ethers, and the major fatty acids and glyceryl ethers were C18:1 (41% of total fatty acids)

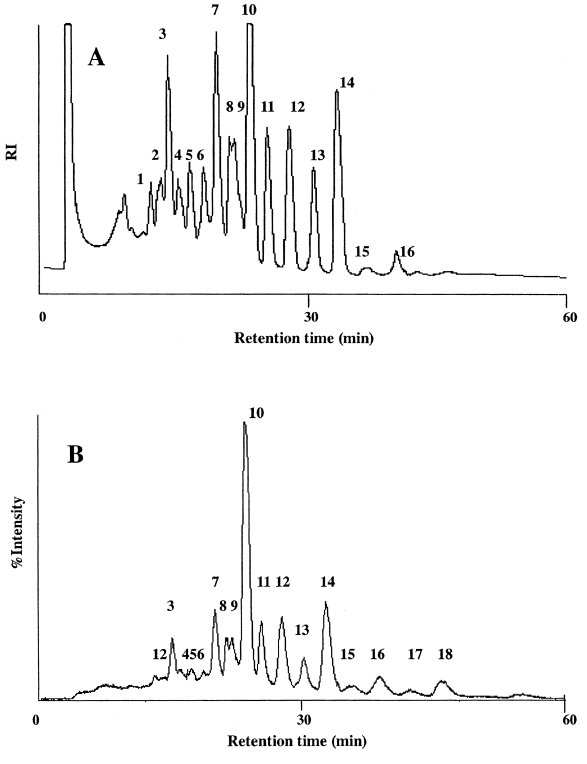

Fig. 4. Refractive index (A) and total ion (B) chromatograms of diacyl glyceryl ethers from *Stromateus stellatus* using the LC–RI–MS method. For chromatographic conditions, see Experimental.

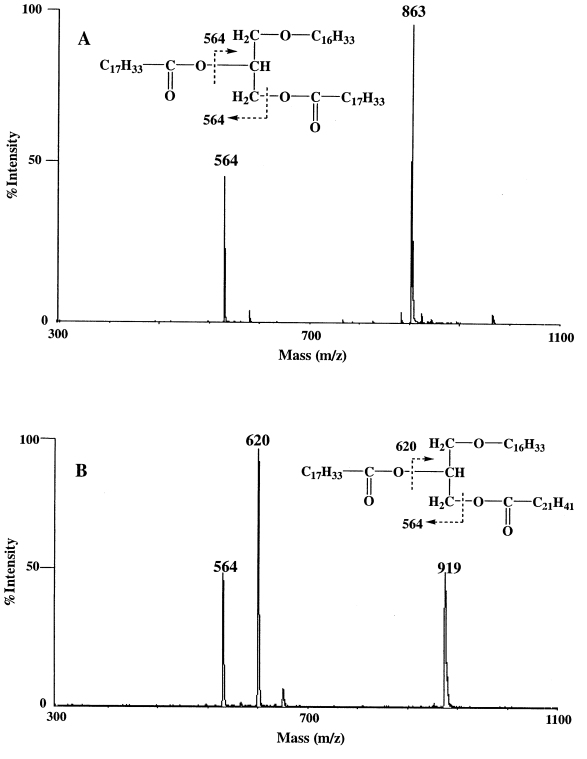

Fig. 5. Mass spectra of Peaks 10 (A) and 14 (B) in diacyl glyceryl ethers from *Stromateus stellatus*.

Table 2
Composition of the diacyl glyceryl ethers (DAGEs) extracted from *Stromateus stellatus*

Peak number	Retention time (min)	DAGE	*m/z*	ECN[a]	(%)[b]
1	13.2	*O*-14:0–18:1–22:6	881, 581, 536	40	1.8
2	14.3	*O*-14:0–18:1–20:5	853, 554, 536	40	2.7
3	15.0	*O*-16:0–18:1–22:6	909, 610, 564	42	7.9
4	16.0	*O*-18:1–16:0–20:5	883, 610, 564	42	3.2
		O-16:0–16:0–22:6	883, 610, 538	42	
5	17.3	*O*-18:0–18:1–22:6	937, 638, 592	44	3.6
6	18.7	*O*-18:0–18:1–20:5	909, 612, 592	44	4.1
		O-18:0–16:0–22:6	911, 638, 566	44	
7	20.3	*O*-14:0–18:1–18:1	835, 536	46	11.1
8	21.6	*O*-14:0–16:0–18:1	809, 536, 510	46	4.4
9	22.1	*O*-18:1–18:1–18:1	889, 590	48	4.7
10	23.7	*O*-16:0–18:1–18:1	863, 564	48	22.7
11	25.2	*O*-16:0–16:0–18:1	837, 564, 538	48	6.5
12	27.7	*O*-18:0–18:1–18:1	891, 592	50	7.6
13	37.1	*O*-16:0–18:0–18:1	865, 566, 564	50	5.5
		O-18:1–18:1–22:1	945, 646, 590	52	
14	32.6	*O*-16:0–18:1–22:1	919, 620, 564	52	11.1
15	35.6	*O*-16:0–18:1–24:1	947, 648, 564	54	0.3
16	38.8	*O*-16:0–22:1–22:1	975, 620	56	1.2

[a] ECN, equivalent carbon number.
[b] The composition (%) of DAGE species was estimated based on their area on the RI chromatogram.

and 1-*O*-hexadecylglycerol (57% of total glyceryl ethers), respectively.

The composition of the DAGE molecular species obtained by LC–RI–MS analysis was consistent with the GLC data.

3.4. Relationship between the retention time and chemical structure of DAGE

The relationship between the Rt and the chemical structure of DAGEs is discussed based on the LC–RI–MS analysis of DAGEs from the deep-sea teleost fish. The Rt is longer as the carbon chain length of the glyceryl ether increases, e.g., the Rt of *O*-18:0–18:1–18:1 was 27.7 min, which was longer than the Rts of *O*-16:0–18:1–18:1 (23.7 min) and *O*-14:0–18:1–18:1 (20.3 min), as shown in Table 2. A similar relationship was observed between the Rt and fatty acids of DAGE. The Rt of *O*-16:0–18:1–24:1 was 35.6 min, which was longer than the Rts of *O*-16:0–18:1–22:1 (32.6 min) and *O*-16:0–18:1–18:1 (23.7 min). The carbon chain length of the fatty acid increased the Rt of the DAGE. In contrast, an increase in the number of double bonds in glyceryl ethers shortens the Rt of the DAGE, e.g., the Rt of *O*-18:1–18:1–18:1 was 22.1 min, which was shorter than that of *O*-18:0–18:1–18:1. Similarly, the Rt of DAGE is shortened by the presence of a double bond in the fatty acid. The Rt of *O*-16:0–18:1–18:1 (23.7 min) was shorter than that of *O*-16:0–18:0–18:1 (30.1 min), as shown in Table 2. Therefore, the equivalent carbon number (ECN) that was proposed in the HPLC analysis of TAG [23,24] was estimated for DAGEs from *Stromateus stellatus*. The ECN was obtained by subtracting twice the number of double bonds from the total carbon number in the DAGE molecule. As shown in Table 2, the ECN ranged from 40 to 56 for DAGEs from *Stromateus stellatus*, and the Rt increased with increasing ECN. This relationship was similar to that in TAG. DAGE molecular species could be characterized by the ECN.

4. Conclusion

A method to identify and quantify DAGE molecular species using LC–RI–MS was developed. The

LC–RI–MS method showed good separation and quantification of synthetic DAGEs. MS data gave direct and important information concerning the fatty acids and glyceryl ethers in DAGEs. When this method was applied to DAGEs extracted from the muscle of *Stromateus stellatus*, 19 molecular species of DAGE were identified and quantified. The LC–RI–MS method is effective for the characterization and quantification of molecular species of DAGEs, as contained in marine animals.

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