An Improved Method for Determining the Composition of FFA in Red Tide Flagellates by RP-HPLC with Fluorescence Detection

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ABSTRACT: An improved method for determining the composition of FFA in red tide flagellates by HPLC with fluorescence detection is described. For this purpose, total lipids from *Heterosigma akashiwo, Chattonella antiqua,* and *C. marina* were allowed to react with 9-anthryldiazomethane, then the resulting 9-anthrylmethyl esters of FFA were analyzed without any purification by RP-HPLC on a highly efficient C18 column (Superspher 100 RP-18e, 25 cm × 4 mm i.d., 4 μm particle size; Merck, Darmstadt, Germany). Clear separations of long-chain saturated and unsaturated FFA, including 14:0 and 16:1, which were major components in the flagellates and were unresolved on a previously used C18 column, were achieved by a stepwise gradient elution using acetonitrile, water, and propan-2-ol. Two characteristic FA, 18:5n-3 and *trans*-16:1n-13, whose behaviors on RP-HPLC had not been reported previously, were also clearly separated from the other FFA. The FFA compositions of the flagellates determined by HPLC were in good agreement with those obtained by GLC. The present method is simple and sensitive, and would be widely applicable for compositional analysis of microalgal FFA.

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KEY WORDS: 9-Anthryldiazomethane, *Chattonella antiqua, Chattonella marina*, fluorescence detection, free fatty acid, *Heterosigma akashiwo*, reversed-phase HPLC.

FFA produced by marine microalgae causing red tides, such as the raphidophyte flagellates *Heterosigma akashiwo*, *Chattonella antiqua,* and *C. marina*, have been implicated in the causative substances that damage the epithelial tissues of fish gills (1,2). Recently, we found that the FFA in *C. marina* are released mainly from monogalactosylglycerols (MGDG) by galactolipase in the alga, where the FFA composition was determined by open-tubular GLC of the methyl esters (3). Although open-tubular GLC has been widely used for determining the composition of FFA in biological samples, it is usually necessary to isolate FFA from crude lipids before methyl esterification or to purify the methyl esters before GLC analysis, which is time-consuming and laborious. In addition, GLC has a limited sensitivity, which usually requires several hundred micrograms of FFA for the analysis.

9-Anthryldiazomethane (ADAM) reacts highly specifically with FFA in crude lipids without preliminary sample purification, catalysts, or heating to give 9-anthrylmethyl ester derivatives, which are highly sensitive and clearly resolved on RP-HPLC with fluorescence detection (4,5). Recently, Suzuki and Matsuyama (6) reported the FFA compositions of several species of the red tide flagellates, including *H. akashiwo* and *C. antiqua*. The FFA were extracted with *n*-hexane from crude lipid extracts dissolved in 80% methanol, then derivatized with ADAM and separated on RP-HPLC on a C18 column (Develosil ODS-5, 25 cm × 4.6 mm i.d., 5 μm particle size; Nomura Chemical, Seto, Japan). Although good separations were achieved for 12 saturated and unsaturated FFA, no resolution of myristic (14:0) and hexadecenoic (16:1) acids, which were major components in the flagellates, was obtained under the conditions employed. Thus, the overlapped components were collected by preparative HPLC and then rechromatographed by RP-HPLC on a cyano column (Capcell Pak CN SG 120, 25 cm × 4.6 mm i.d, 5 μm particle size; Shiseido, Tokyo, Japan), which gave good resolution of 14:0 and 16:1. In this study, we found that there is no need to isolate or purify FFA from total lipids of the flagellates, and the derivatives of saturated and unsaturated FFA, including 14:0 and 16:1, are clearly separated on a highly efficient C18 column (Supersphere RP-18e, 25 cm × 4 mm i.d., 4 μm particle size) by a stepwise gradient elution using acetonitrile, water, and propan-2-ol.

This paper describes an improvement and simplification of the Suzuki and Matsuyama method (6) for determining the FFA compositions of red tide flagellates by RP-HPLC with fluorescence detection.

EXPERIMENTAL PROCEDURES

FA standards. Authentic standards of myristic (14:0), palmitic (16:0), palmitoleic (16:1n-9), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), and α-linolenic (18:3n-3) acids were purchased from Sigma (St. Louis, MO). γ-Linolenic (18:3n-6) and arachidonic (20:4n-6) acids were products of Idemitsu Petrochemical (Tokyo, Japan). EPA (20:5n-3) and DHA

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(22:6n-3) were products of Nippon Chemical Feed (Hakodate, Japan). Octadecatetraenoic acid (18:4n-3) was prepared from fish oils by silver nitrate TLC (7,8). *trans*-3-Hexadecenoic (*trans*-16:1n-13) and docosapentaenoic (22:5n-3) acids were prepared from phosphatidylglycerols in spinach leaves (3,9) and PC in salmon eggs (10), respectively. A mixture of FA containing hexadecadienoic (16:2n-6) and hexadecatrienoic (16:3n-3) acids was prepared from MGDG of the green alga *Chlorella vulgaris* (11).

Chemicals. ADAM was a product of Funakoshi (Tokyo, Japan). Acetonitrile, propan-2-ol, and methanol were of HPLC grade (Kanto Chemicals, Tokyo, Japan). Kieselgel 60 GF glass-backed plates (20 × 20 cm, 0.25 mm gel thickness) for TLC were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were of reagent grade or better quality and were also obtained from Kanto Chemicals.

Algal cultures. *Heterosigma akashiwo* (NIES-4), *C. antiqua* (NIES-1), and *C. marina* (NIES-3) were obtained from the National Institute for Environmental Studies (Tsukuba, Japan). These flagellates were grown in 2 L of a sterile f/2 medium at 20°C under 4000 lux of cool-white fluorescent illumination on a 12 h light/12 h dark cycle (3). Cells (1.0×10^5 cells/mL for *H. akashiwo* in the early stationary phase, 2.0×10^3 cells/mL for *C. antiqua* in the middle stationary phase, 2.0×10^4 cells/mL for *C. marina* in the early stationary phase) were harvested by centrifugation (1,500 × *g*, 10 min).

Derivatization. Total lipids, including FFA, which were extracted by the method of Bligh and Dyer (12), were subjected to derivatization with ADAM for HPLC (13). In brief, to 50–100 μg of total lipids containing 3–14% FFA, 0.1 mL of 0.01–0.1% (wt/vol) ADAM in methanol was added. After maintaining the reaction mixture for 1 h at room temperature, 10 μL was injected into the HPLC column as described below.

RP-HPLC. HPLC analysis was carried out with a Waters LC Module 1 (Waters, Milford, MA) equipped with a C18 column (Superspher RP-18e, 25 cm × 4 mm i.d., Merck). The mobile phase was acetonitrile/water (98:2, vol/vol) for 30 min, and was changed by a linear gradient from acetonitrile/propan-2-ol (98:2, vol/vol) to acetonitrile/propan-2-ol (50:50, vol/vol) over 20 min at a flow rate of 0.5 mL/min. The column temperature was 22°C. Peaks were monitored with a Waters 474 Scanning Fluorescence Detector. The excitation and emission wavelengths were set at 365 and 412 nm, respectively (13).

GLC. GLC analysis of methyl esters was performed with a Shimadzu GC-14A gas chromatograph equipped with an FID (Shimadzu, Kyoto, Japan). FFA were derivatized into methyl esters with 5% HCl/methanol (7), then resolved on an Omegawax 320 column (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA). The column temperature was programmed from 180 to 240°C at 1°C/min. The carrier gas was helium at a flow rate of 1.2 mL/min, and the split ratio was 50:1 at room temperature. Injector and detector temperatures were maintained at 250 and 260°C, respectively. Peak areas were measured on a Chromatopac C-R6A (Shimadzu). FA were identified by comparison with the known FA from marine organisms, as described previously (8).

RESULTS AND DISCUSSION

Resolution of a mixture of FA standards. Figure 1 shows the RP-HPLC separation of the 9-anthrylmethyl esters of saturated and unsaturated FA standards on Superspher RP-18e. Most of the FFA containing 14:0 and 16:1n-9, which were overlapped on the Develosil ODS column (6), were clearly resolved by a gradient elution using a solvent system consisting of acetonitrile, water, and propan-2-ol. FA having the same ECN (equivalent carbon number: carbon number of acyl group – 2 × number of double bonds) values, that is, 16:3n-3, 18:4n-3, 20:5n-3, and 22:6n-3 (ECN = 10); 16:2n-6, 18:3n-3 + 18:3n-6, and 20:4n-6 + 22:5n-3 (ECN = 12); 16:1n-9, 18:2n-6, 14:0, and *trans*-16:1n-13 (ECN = 14); 18:1n-9 and 16:0 (ECN = 16); and 18:0 (ECN = 18) were eluted in this order.

Figure 2 shows the resolution of the 9-anthrylmethyl esters of a mixture of 14:0, 16:1n-9, and 18:2n-6 obtained on the Superspher RP-18e column using different mobile phases. The latter two components overlapped partially by a gradient elution using a mobile phase consisting of acetonitrile, water, and propan-2-ol (Fig. 1). When acetonitrile/water (98:2, vol/vol) was used as the mobile phase, 18:2n-6 was eluted between 16:1n-9 and 14:0 with a retention time close to 16:1n-9 (Fig. 2A). When methanol/water (98:2, vol/vol) was used as the mobile phase, on the other hand, 14:0 was eluted between 16:1n-9 and 18:2n-6 (Fig. 2B). Faster elutions of unsaturates than saturates were also observed for the anthrylmethyl esters of 16:0 and 18:1n-9 having the same ECN values (chromatograms not shown), whose separation has already been reported (13–15). The elution orders observed, however, are different from those reported by Roemen and Van Der Vusse (5) and Shimomura *et al*. (15), who observed that the anthrylmethyl esters of 14:0, 16:1, 18:2, 16:0, and 18:1 eluted in this

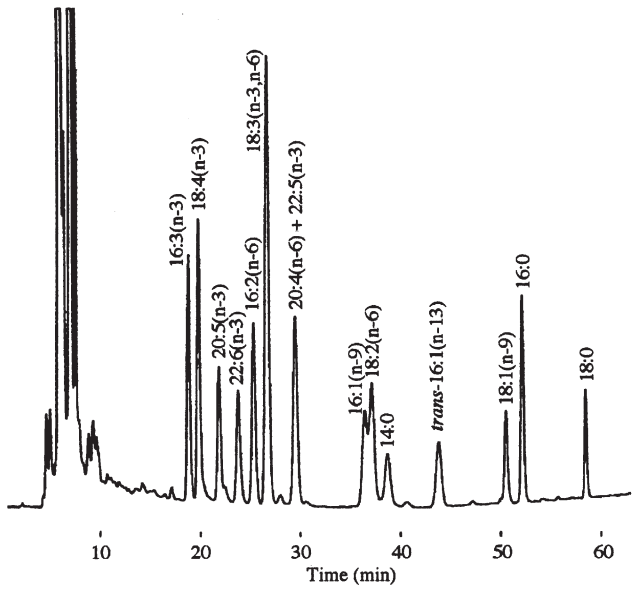

FIG. 1. RP-HPLC resolution of a mixture of FFA standards as 9-anthrylmethyl esters. HPLC conditions as given in text.

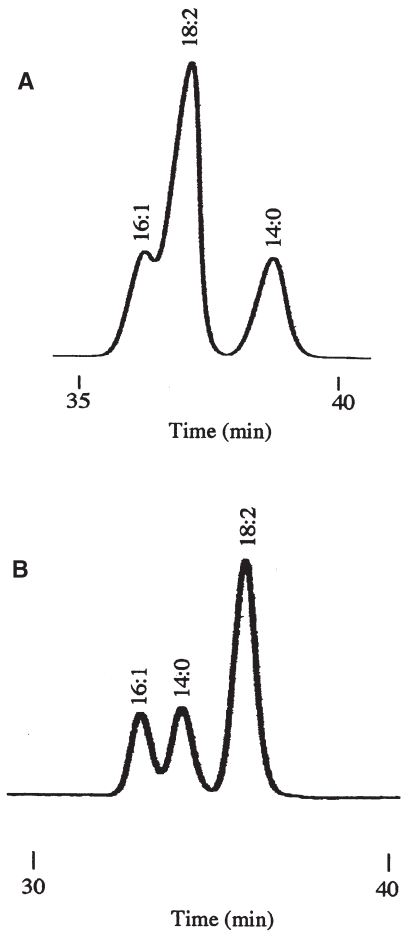

FIG. 2. RP-HPLC resolution of a mixture of 14:0, 16:1n-9, and 18:2n-6 as 9-anthrylmethyl esters. Mobile phase: (A) acetonitrile/water (98:2, vol/vol); (B) methanol/water (98:2, vol/vol). Other HPLC conditions as given in text.

order on C18 columns using acetonitrile/water (93:7, vol/vol) and methanol/water (94.7:5.3, vol/vol) as the mobile phases, respectively. On the other hand, Yoshida *et al.* (16) observed that the anthrylmethyl esters of 16:1, 14:0, 18:2, 16:0, and 18:1 eluted in this order on a reversed-phase column (TSK-GEL-120T; Tosoh, Tokyo, Japan) using 84% aqueous acetonitrile as the mobile phase. We cannot offer a satisfactory explanation for these differences in the elution order of the FFA derivatives on RP-HPLC, but they were probably caused by small differences in the conditions employed, such as column temperature and water content in the mobile phases. Further work on the HPLC behavior of the anthrylmethyl ester derivatives is in progress.

Although clear resolution among 16:1n-9, 14:0, and 18:2n-6 was achieved using methanol/water (98:2, vol/vol) as the mobile phase (Fig. 2B), poorer resolution of the other components containing highly unsaturated FA such as 20:5n-3 and 22:6n-3 was obtained (chromatograms not shown). In this study, therefore, an acetonitrile/water system was employed for analysis of the FFA in the red tide algae as described below.

Elution profiles of the FFA in H. akashiwo *and* C. antiqua. Figure 3 shows the RP-HPLC separation of the 9-anthrylmethyl esters of the FFA from *H. akashiwo* and *C. antiqua* on the Superspher RP-18e column. Main components including 14:0 and 16:1n-9 were clearly separated in a single HPLC run. Peaks were identified by comparing the retention times with those obtained for the FFA standards (see Fig. 1). Although the anthrylmethyl esters of 14:0 and 16:1 in synthetic and biological samples have already been observed on RP-HPLC (5,15,16), there are no reports for marine microalgae having complex FFA compositions. A significant amount of 18:5n-3, which has been proposed as a biological marker for dinoflagellates in the marine food chain, is known to exist in glycoglycerolipids of certain marine microalgae, including *H. akashiwo* (17–19), although this acid was not identified in the previous study, nor were 15:0, *trans*-16:1n-13, 16:2n-6, 16:3n-3, 18:3n-6, and 22:5n-3 (6). In this study, the 18:5n-3 from *H. akashiwo*, which eluted ahead of 16:3n-3 (ECN = 10), was tentatively identified on the basis of its ECN value (ECN = 8). This identification was also supported by GLC analysis of the FAME. A characteristic FA, *trans*-16:1n-13, which would have been released from phosphatidylglycerols by galactolipase (3), was also detected in the FFA from all the algae examined. Several isomers of 16:1 (*cis* n-3, n-4, n-5, n-7, n-9, n-11, and *trans* n-13) have been found in the lipids of

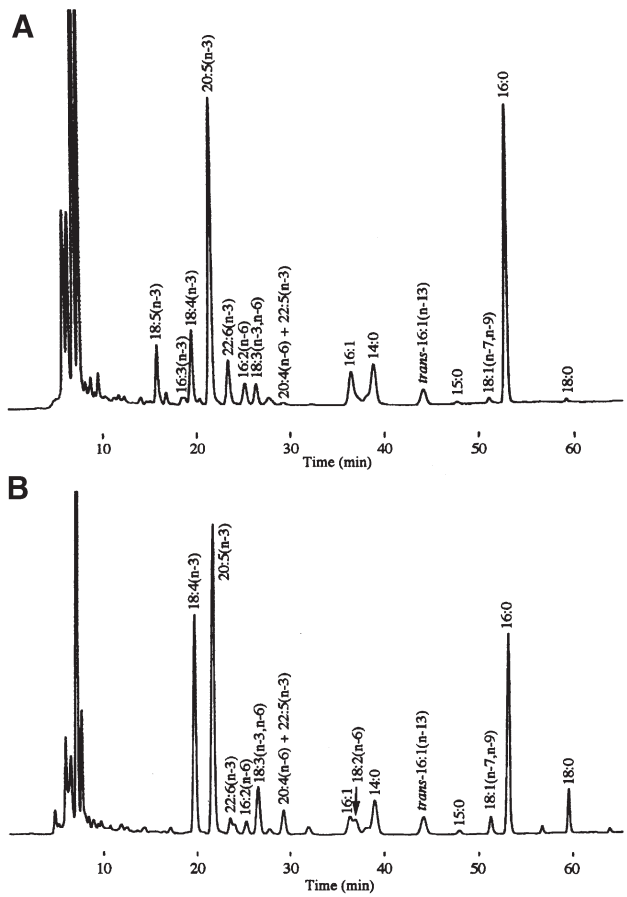

FIG. 3. RP-HPLC profiles of the FFA in *Heterosigma akashiwo* (A) and *Chattonella antiqua* (B). HPLC conditions as given in text.

TABLE 1
Chromatographic Parameters of the 9-Anthrylmethyl Esters of FA

FA[a]	ECN[b]	V_R[c]	k'[d]	α[e]
18:5n-3	8	8.00	2.99	
				1.19
16:3n-3	10	9.53	3.56	
				1.03
18:4n-3	10	9.86	3.68	
				1.10
20:5n-3	10	10.87	4.06	
				1.09
22:6n-3	10	11.84	4.42	
				1.07
16:2n-6	12	12.70	4.74	
				1.05
18:3n-3, 18:3n-6	12	13.36	4.98	
				1.11
20:4n-6, 22:5n-3	12	14.78	5.51	
				1.24
16:1	14	18.42	6.87	
				1.02
18:2n-6	14	18.75	6.99	
				1.05
14:0	14	19.65	7.33	
				1.14
trans-16:1n-3	14	22.33	8.33	
				1.11
15:0	15	24.83	9.26	
				1.16
18:1n-7, 18:1n-9	16	28.90	10.78	
				1.09
16:0	16	31.65	11.81	
				1.65
18:0	18	52.13	19.45	

[a]FFA from *Heterosigma akashiwo* and *Chattonella antiqua* were used to obtain these data.
[b]ECN, equivalent carbon number (total acyl carbon numbers − 2 × number of double bonds).
[c]V_R is retention volume (mL) corrected by subtracting the column void volume (2.68 mL). Data were obtained by an isocratic elution with acetonitrile/water (98:2, vol/vol) at 0.5 mL/min. Other HPLC conditions as given in text.
[d]k', capacity factor.
[e]α, separation coefficient between neighboring FA.

H. akashiwo by GLC of the methyl esters (19). Because no reference standards exist, however, we could not clarify the HPLC behavior of the anthrylmethyl esters of the *cis*-16:1 isomers except for n-7 and n-9, which eluted ahead of 18:2n-6 with a complete overlapping.

Clear separations of major saturated and unsaturated FFA were also observed for *C. marina* under the same HPLC conditions as those employed for *H. akashiwo* and *C. antiqua* (chromatograms not shown).

Interrelationships of the retention data. Table 1 gives the chromatographic parameters of FFA as 9-anthrylmethyl esters on Superspher RP-18. The separation coefficients (α) for the difference of two carbon numbers between 14:0 and 16:0 and between 16:0 and 18:0 were 1.61 and 1.65, respectively. The α values for the difference of one double bond for 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, and 18:5n-3 were 1.80, 1.54, 1.40, 1.35, and 1.23, respectively, showing clearly the decreases with the increasing number of double bonds. A high α value (α = 1.21) comparable to that of 18:4n-3 and 18:5n-3 (α = 1.23) was obtained for *cis*-16:1(n-9 + n-7) and *trans*-16:1n-13, having the same carbon number, which had close retentions on GLC (3,18). Thus, the *trans*-16:1n-13 in the flagellate FFA was clearly separated from other components on RP-HPLC (see Fig. 3). RP-HPLC of the 9-anthrylmethyl ester derivatives would be useful for detection of *trans*-16:1n-13 in biological samples.

Figure 4 shows the linear relationship in logarithmic retention volume (V_R) against the acyl carbon number in each homologous series of FFA as 9-anthrylmethyl esters. The straight lines are not parallel, and the distance between the lines progressively increases with increasing carbon number. This indicates that the α values increase with increasing carbon number under the conditions used. Thus, the following equations can be obtained for the C_{16} and C_{18} homologous series.

$$\log V_R(16:0) = \log V_R(16:1) + 0.25 \quad [1]$$
$$\log V_R(16:1) = \log V_R(16:2) + 0.18 \quad [2]$$
$$\log V_R(16:2) = \log V_R(16:3) + 0.15 \quad [3]$$
$$\log V_R(18:0) = \log V_R(18:1) + 0.27 \quad [4]$$
$$\log V_R(18:1) = \log V_R(18:2) + 0.20 \quad [5]$$

TABLE 2
Composition of FFA in Red Tide Flagellates (mol%)

	Heterosigma akashiwo			*Chattonella antiqua*			*Chattonella marina*	
FFA	HPLC	GLC	HPLC[a]	HPLC	GLC	HPLC[a]	HPLC	GLC
14:0	9.3	7.1	4.9	7.5	6.6	3.5	10.3	6.2
15:0	0.3	0.3		0.5	0.5		0.7	0.8
16:0	26.7	29.7	20.2	18.4	18.9	19.6	27.2	27.5
18:0	0.2	0.3	2.8	3.0	1.2	1.4	0.4	0.5
20:0	ND[b]	ND	0.5	ND	ND	0.2	ND	0.1
ΣSaturates	36.5	37.4	28.4	29.4	27.2	24.7	38.6	35.1
16:1	7.0	10.6	2.7	2.8	4.6	6.5	13.3	12.3
trans-16:1n-13	2.8	1.0		3.3	0.9		1.9	2.0
18:1n-7	0.6	0.3	ND	1.8	ND	0.7	1.3	0.7
18:1n-9		0.4			1.4			0.6
ΣMonounsaturates	10.4	12.3	2.7	7.9	6.9	7.2	16.5	15.6
16:2n-6	3.0	2.1		1.5	0.2		2.2	1.0
16:3n-3	0.7	0.5		ND	ND		0.3	0.1
18:2n-6	ND	0.4	0.2	2.3	2.3	3.0	0.7	2.4
18:3n-3	2.5	2.3	3.3	5.5	5.1	6.6	3.3	2.0
18:3n-6		0.1			0.6			1.1
18:4n-3	7.5	6.9	4.4	19.7	18.1	20.4	9.2	8.6
18:5n-3	5.5	2.9		ND	ND		ND	ND
20:4n-6	0.2	0.2	1.7	2.7	3.1	5.8	5.3	5.5
22:5n-3		0.2			0.1			1.3
20:5n-3	29.1	29.2	57.6	29.5	32.0	29.6	22.9	23.9
22:6n-3	4.6	3.7	1.7	1.5	1.5	2.7	1.0	0.9
ΣPolyunsaturates	53.1	48.5	68.9	62.7	63.0	68.1	44.9	46.8
Others		1.8			2.9			2.5

[a]Data from Reference 6. The position of the double bonds was not specified.
[b]ND, not detected. HPLC conditions as given in text.

$$\log V_R(18:2) = \log V_R(18:3) + 0.16 \quad [6]$$
$$\log V_R(18:3) = \log V_R(18:4) + 0.15 \quad [7]$$
$$\log V_R(18:4) = \log V_R(18:5) + 0.11 \quad [8]$$

These relationships would be helpful for tentative identification of the 9-anthrylmethyl esters of FFA in biological samples. Actually, the 18:5n-3 in *H. akashiwo* was confirmed using these relationships (see Fig. 3). The slope of the line connecting the points of 18:4n-3 and 20:4n-6 was slightly higher than the others. This suggests that the double bond positional isomers of 18:4n-3 and 18:4n-6 and/or 20:4n-3 and 20:4n-6 may be separable on RP-HPLC, although no separation was observed for 18:3n-3 and 18:3n-6 under the conditions employed.

Compositional analysis of the FFA in red tide flagellates. Table 2 compares the FFA compositions of *H. akashiwo*, *C. antiqua,* and *C. marina* obtained by HPLC and GLC. A fair agreement between the compositions obtained by the two analytical methods employed in this study indicates the reliability for quantitative analysis of FFA as 9-anthrylmethyl esters by RP-HPLC with fluorescence detection. The FFA composition of *C. antiqua* was in good agreement with the previous study using HPLC (6), whereas for *H. akashiwo* it was quite different, particularly for 20:5n-3 (Table 2). The differences between FA compositions might be caused by different strain and/or culture conditions (20).

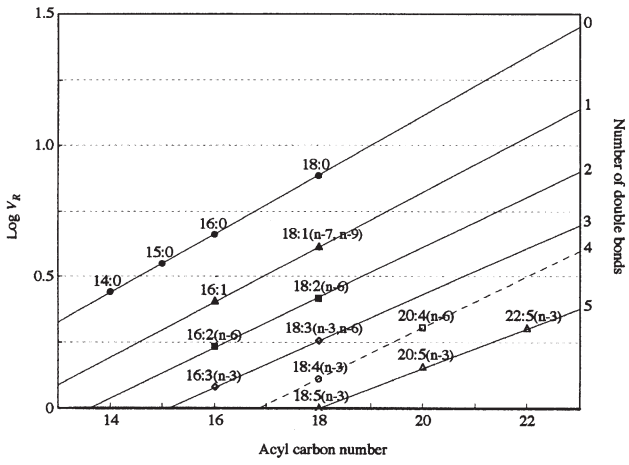

FIG. 4. Plots of carbon number vs. logarithm of the relative retention volume (V_R) of the 9-anthrylmethyl esters of FFA. Mobile phase: acetonitrile/water (98:2, vol/vol). Other HPLC conditions as given in text.

In conclusion, this study provides an improved HPLC method for determining the composition of the FFA in red tide flagellates such as the *Heterosigma* and *Chattonella* species. The method using RP-HPLC with fluorescence detection on a highly efficient C_{18} column is simpler than that reported previously (6) and gives more detailed FFA compositions, which would be helpful in promoting work on the production and physiological importance of FFA in red tide flagellates.

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