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Hydrogen isotopic fractionations during desaturation and elongation associated with polyunsaturated fatty acid biosynthesis in marine macroalgae

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Abstract

Compound-specific hydrogen isotopic compositions (δD) of saturated, monounsaturated and polyunsaturated fatty acids have been determined for natural marine macroalgae including two brown algae (Heterokontophyta) and two red algae (Rhodophyta). δD values of individual fatty acids from four macroalgae exhibit a wide variation ranging from $-189‰$ to $+48‰$. Generally, stearic (18:0), arachidic (20:0) and behenic acids (22:0) are much more enriched in D by up to $\sim 180‰$ relative to myristic (14:0), palmitic (16:0), octatetraenoic [18:4($n-3$)] and eicosapentaenoic acids [20:5($n-3$)]. Other fatty acids such as oleic [18:1($n-9$)], lenoleic [18:2($n-6$)] and linolenic acids [18:3($n-3$)] fall isotopically between these fatty acids. This wide δD variation of fatty acids is probably explained by the hydrogen isotopic fractionation during desaturation being much larger than that during elongation in the network of polyunsaturated fatty acid biosynthesis. A large hydrogen isotopic fractionation during desaturation may cause D-enrichment in the remaining hydrogen of the residual fatty acids, which could be controlled by the relative flux into their desaturates.

Keywords: Marine macroalgae; Hydrogen isotopic composition; Hydrogen isotopic fractionation; Lipid biosynthesis; Desaturation; Elongation; Polyunsaturated fatty acid

1. Introduction

Knowledge of hydrogen isotopic compositions and fractionations of individual lipid biomolecules in primary producers such as algae and higher plants is biologically and geochemically significant (e.g., Hayes, 2001). Individual lipid biomolecules have various hydrogen isotopic compositions, which can provide useful information on the biochemical processes involved in their biosynthetic pathways (e.g., Sessions et al., 2002; Chikaraishi et al., 2004). Distinct hydrogen isotopic compositions (or fractionations) among organisms are critical in order to estimate biological sources for the lipid molecules in natural samples such as sediments (Sauer et al., 2001). In addition, an understanding of hydrogen isotopic fractionations between lipid biomolecules and environmental water would facilitate reconstruction of hydrological cycles in paleoenvironmental studies. (Xie et al., 2000; Huang et al., 2002).

Although carbon isotopic fractionations related to various lipid biosyntheses have been reported in many previous studies (e.g., DeNiro and Epstein, 1977; Monson and Hayes, 1982; Hayes, 1993), their hydrogen

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isotopic fractionations have not been well clarified yet. Recently, some hydrogen isotopic fractionations during the lipid biosyntheses have been reported (e.g., Sessions et al., 1999, 2002; Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004), using compound-specific hydrogen isotope analysis (Burgøyne and Hayes, 1998; Hilkert et al., 1999). For example, Sessions et al. (2002) reported hydrogen isotopic fractionations of lipid biomolecules such as sterols and hopanols in methane-oxidizing bacterium, and suggested that 31.4% of hydrogen in lipid biomolecules were derived from methane. Chikaraishi et al. (2004) discussed hydrogen isotopic fractionations of various lipid molecules associated with their biochemical processes such as hydrogenation and hydrogen-exchange reactions with respect to three typical lipid biosynthetic pathways including acetogenic, mevalonaic-acid and methylerythritol phosphate within a single higher plant (*Cryptomeria japonica*). Generally, hydrogen isotopic compositions of lipid biomolecules should depend on isotopically distinct hydrogen sources and isotope effects during hydrogen-involving reactions such as hydrogenation and dehydration in the their biosynthetic pathways (Sessions et al., 1999, 2002; Chikaraishi et al., 2004).

Fatty acids are the most abundant lipid biomolecules as major constituents of cell membrane in organisms, and will affect a large influence on hydrogen isotopic compositions of sedimentary organic matters. However, hydrogen isotopic compositions of fatty acids with respect to various degrees of carbon lengths and unsaturations have not been characterized yet. The purpose of this study is to investigate hydrogen isotopic compositions of saturated, monounsaturated and polyunsaturated fatty acids in natural marine macroalgae (brown algae: *Sargassum filicinum* and *Undararia pinnatifida*, and red algae: *Binghamia californica* and *Gelidium japonica*). Furthermore, this study will clarify the mechanism of hydrogen isotopic fractionations during desaturation and elongation associated with polyunsaturated fatty acid biosynthesis, which provide information to understand hydrogen isotopic distributions of individual fatty acids in biological and geochemical samples.

2. Results and discussion

2.1. Molecular distributions

As summarized in Table 1, saturated (**1**–**5**), monounsaturated (**6**–**10**) and polyunsaturated fatty acids (**11**–**22**) are identified in four marine macroalgae. Palmitic acid (16:0) (**2**) is the most abundant, accounting for 55.5%, 45.4%, 42.3% and 50.3% to the total fatty acids in *S. filicinum*, *U. pinnatifida*, *B. californica* and *G. japoniuca*, respectively. These marine macroalgae contain oleic [18:1($n-9$)] (**8**), linoleic [18:2($n-6$)] (**12**), α-linolenic [18:3($n-3$)] (**15**), arachidonic [20:4($n-6$)] (**20**) and eicosapentaenoic acids [20:5($n-3$)] (**22**), as major mono- and poly-unsaturated fatty acids. These fatty acids have been typically found in marine macroalgae (e.g., Jamieson and Reid, 1972; Demebitsky et al., 1993; Fleurence et al., 1994) as well as microalgae (e.g., Stefanov et al., 1988; Dembitsky et al., 1990; Viso and Marty, 1993; Dunstan et al., 1994). Though small amounts of uncommon 16:1($n-5$) fatty acid (**7**) is also identified in two brown macroalgae, this fatty acid was reported previously in some brown microalgae (Khotimchenko, 1995).

2.2. Compound-specific carbon isotopic compositions

Carbon isotopic compositions (δ^{13}C, ‰ relative to Peedee Belemnite, PDB) of individual fatty acids are summarized in Table 1. Individual fatty acids of brown algae (*S. filicinum* and *U. pinnatifida*) exhibit δ^{13}C values ranging from −20.6‰ to −18.0‰, being a little depleted in ^{13}C compared to those of red algae (*B. californica* and *G. japoniuca*) with δ^{13}C values ranging from −17.8‰ to −15.9‰. No significant difference in δ^{13}C values among individual fatty acids within a single species is found in these marine macroalgae, in which δ^{13}C variation is ±0.7‰ for *S. filicinum*, ±1‰ for *U. pinnatifida*, ±0.9‰ for *B. californica* and ±0.5‰ for *G. japoniuca*. This suggests that ^{13}C-discrimination dependent on the carbon number and degree of unsaturation cannot occur in fatty acids of marine macroalgae.

2.3. Compound-specific hydrogen isotopic compositions

Hydrogen isotopic compositions (δD, relative to Standard Mean Ocean Water, SMOW) of individual fatty acids are also summarized in Table 1. In contrast to δ^{13}C values, δD values of individual fatty acids varied widely within a single species, in which the range is −189‰ to −29‰ for *S. filicinum*, −182‰ to −22‰ for *U. pinnatifida*, −175‰ to −42‰ for *B. californica* and −139‰ to +48‰ for *G. japoniuca*. Generally, stearic (18:0) (**3**), arachidic (20:0) (**4**) and behenic acids (22:0) (**5**) are much more enriched in D by up to ~180‰ relative to myristic (14:0) (**1**), 16:0 (**2**), octatetraenoic acid [18:4($n-3$)] (**19**) and 20:5($n-3$) (**22**). Other fatty acids such as 18:1($n-9$) (**8**), 18:2($n-6$) (**12**) and 18:3($n-3$) (**15**) fall isotopically between these fatty acids. Though δD values of individual fatty acids from *G. japoniuca* are relatively enriched in D by ~50‰ compared with those of other macroalgae, the isotopic trend among individual fatty acids within an alga is consistent with that for other three macroalgae. This δD distribution can be explained by a similar kinetic isotope effect during polyunsaturated fatty acid biosynthesis among four macroalgae. The wide δD variation (up to 183‰) within a single species is probably due to hydrogen isotopic fractionation during desaturation (see below).

Table 1
Molecular abundance (μmol/dry g) and compound-specific carbon ($\delta^{13}C$, ‰ relative to PDB) and hydrogen isotopic compositions (δD, ‰ relative to SMOW) of individual fatty acids extracted from four marine macroalgae

Compound	Brown algae (Heterokontophyta)						Red algae (Rhodophyta)					
	S. filicinum			*U. pinnatifida*			*B. californica*			*G. japonica*		
Fatty acid	Abundance	$\delta^{13}C$	δD	Abundance	$\delta^{13}C$	δD	Abundance	$\delta^{13}C$	δD	Abundance	$\delta^{13}C$	δD
Saturated												
14:0 (**1**)	1.88	−20.3	−186	1.58	−20.0	−182	2.68	−17.2	−175	1.46	−17.6	−135
16:0 (**2**)	15.39	−20.2	−188	8.66	−20.0	−178	12.96	−17.0	−168	10.43	−17.4	−135
18:0 (**3**)	0.30	−20.5	−72	0.54	−20.0	−22	0.64	−16.5	−42	0.31	−17.7	+48
20:0 (**4**)	0.12	−19.3	−46	0.19			0.14	−16.9	−59	0.01	−17.2	+42
22:0 (**5**)	0.11	−19.8	−29				0.05	−16.6	−62	0.01	−17.0	+19
Monounsaturated												
16:1 (*n* − 7) (**6**)	1.09			0.13			0.39			0.57		
16:1 (*n* − 5) (**7**)	0.03			0.00								
18:1 (*n* − 9) (**8**)	2.07	−20.1	−111	2.21	−19.0	−82	3.34	−16.4	−114	0.81	−17.3	−69
20:1 (*n* − 11) (**9**)	0.39	−19.6	−84	0.08						0.01	−17.8	+25
22:1 (*n* − 13) (**10**)	0.19	−19.1	−56									
Diunsaturated												
16:2 (*n* − 4) (**11**)	0.05						0.07					
18:2 (*n* − 6) (**12**)	0.93	−20.2	−131	0.79	−19.1	−130	5.35	−15.9	−138			
20:2 (*n* − 6) (**13**)	0.22	−19.6	−107				0.11	−16.7	−130			
Triunsaturated												
18:3 (*n* − 6) (**14**)	0.15	−20.4	−169	0.12	−19.6	−147	0.23	−17.0	−145	0.30	−17.3	−83
18:3 (*n* − 3) (**15**)	0.50	−20.6	−88	0.90	−19.8	−73	1.25	−17.3	−95	2.31	−17.5	−107
20:3[a] (**16**) + 20:3 (*n* − 6) (**17**)	0.14	−19.9	−65	0.05	−18.5	−36	0.10	−17.3	−60			
20:3 (*n* − 3) (**18**)				0.00			0.04					
Tetraunsaturated												
18:4 (*n* − 3) (**19**)	0.50	−20.2	−189	1.27	−18.5	−182	1.22	−17.3	−169	1.11	−17.3	−133
20:4 (*n* − 6) (**20**)	2.74	−19.5	−175	1.49	−19.1	−150	0.98	−17.1	−149	2.58	−17.2	−99
20:4 (*n* − 3) (**21**)				0.05	−19.4	−28	0.07	−17.4	−88			
Pentaunsaturated												
20:5 (*n* − 3) (**22**)	0.94	−19.9	−188	1.01	−18.0	−180	0.99	−16.3	−167	0.83	−17.8	−139

[a] 20:3 ($\Delta^{5,11,14}$).

2.4. Hydrogen isotopic fractionations during desaturation and elongation

As shown in Fig. 1, polyunsaturated fatty acid biosynthesis is a complex network composed of a number of desaturation and elongation reactions, in which two hydrogens are removed during each desaturation, and four hydrogens are incorporated during each elongation. Hydrogen isotopic compositions of individual fatty acids should be closely related to the isotope effect and relative flux at a branching point in each hydrogen-involving reaction. Although there is a possibility that δD values of individual fatty acids also depend on the hydrogen exchange by enzymatic catalysis, hydrogen isotopic fractionations on the hydrogens directly affected by desaturation and elongation are probably the most important to understand δD variation of individual fatty acids. A simple model of desaturation and elongation is illustrated in Fig. 2. In the case of a desaturation of A to form B, hydrogen isotopic fractionation could occur between the two hydrogens removed during desaturation and the two hydrogens remaining on residual A (cross in Fig. 2). Similarly, during continuous desaturation of B to form C, hydrogen isotopic fractionation could also occur between the two hydrogens removed during desaturation and the two hydrogens remaining on residual B (triangle in Fig. 2). Isotopic compositions of the removed and remaining hydrogens should be related to the kinetic isotope effect and relative flux at each desaturation process. In addition, in the case of an elongation of A to form E, hydrogen isotopic compositions of E should depend on isotopic mass balance between hydrogens on A (open-circle, cross and triangle in Fig. 2) as a primer and four hydrogens incorporated during elongation (filled-circles in Fig. 2). δD value of the incorporated hydrogen results from the integrated

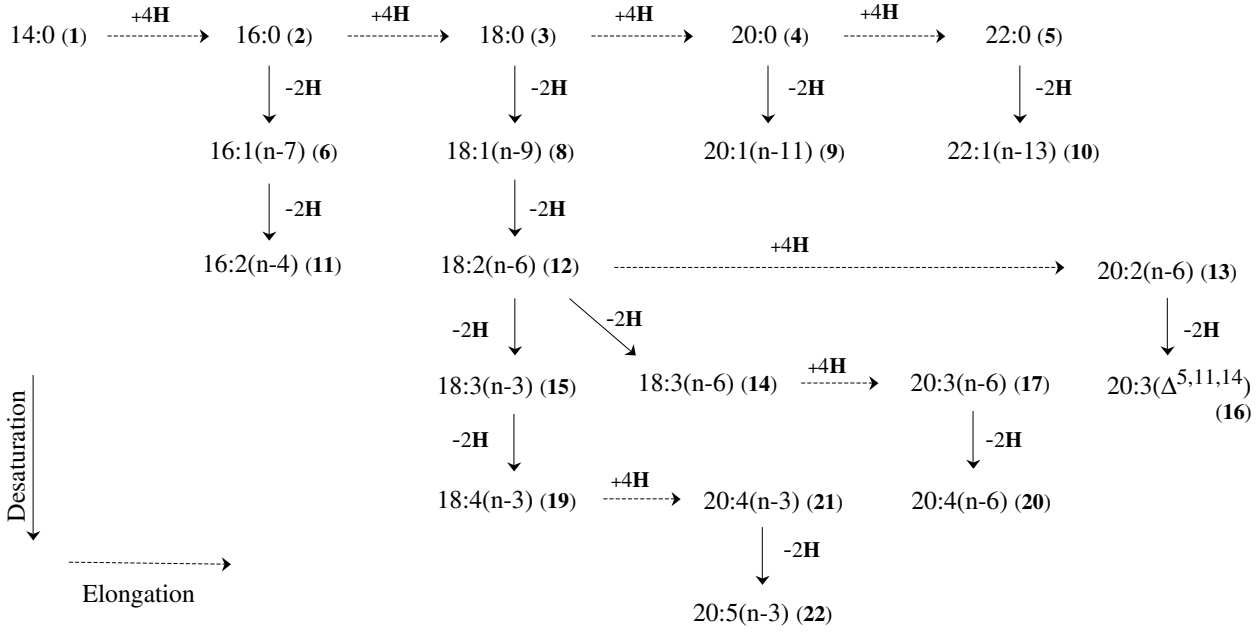

Fig. 1. Major pathway of polyunsaturated fatty acid biosynthesis in marine macroalgae (after Harwood and Jones, 1989; Cohen and Heimer, 1990; Khozin and Cohen, 1996; Shiran et al., 1996; Khozin et al., 1997).

isotopic fractionations during the complex elongation processes such as reduction and dehydration as well as hydrogen-exchange reaction.

For all macroalgae, among fatty acids of 18:0 (**3**) to 18:4($n-3$) (**19**) relating the continuous desaturations, 18:0 (**3**) is the most enriched in D while 18:4($n-3$) (**19**) is the most depleted in D. Other intermediate fatty acids [18:1($n-9$) (**8**), 18:2($n-6$) (**12**) and 18:3($n-3$) (**15**)] fall isotopically between these two fatty acids. For the isotopic fractionation between the removed and remaining hydrogens, the observed δD variation requires that the two hydrogens removed during desaturation must be depleted in D relative to the two hydrogens remaining on the residual point, being enriched in D. The relationship between δD values and relative abundance (mol%) for 18:0 (**3**), 18:1($n-9$) (**8**), 18:2($n-6$) (**12**) and 18:3($n-3$) (**15**) is shown in Fig. 3, which clearly indicates that low abundant fatty acids are more en-

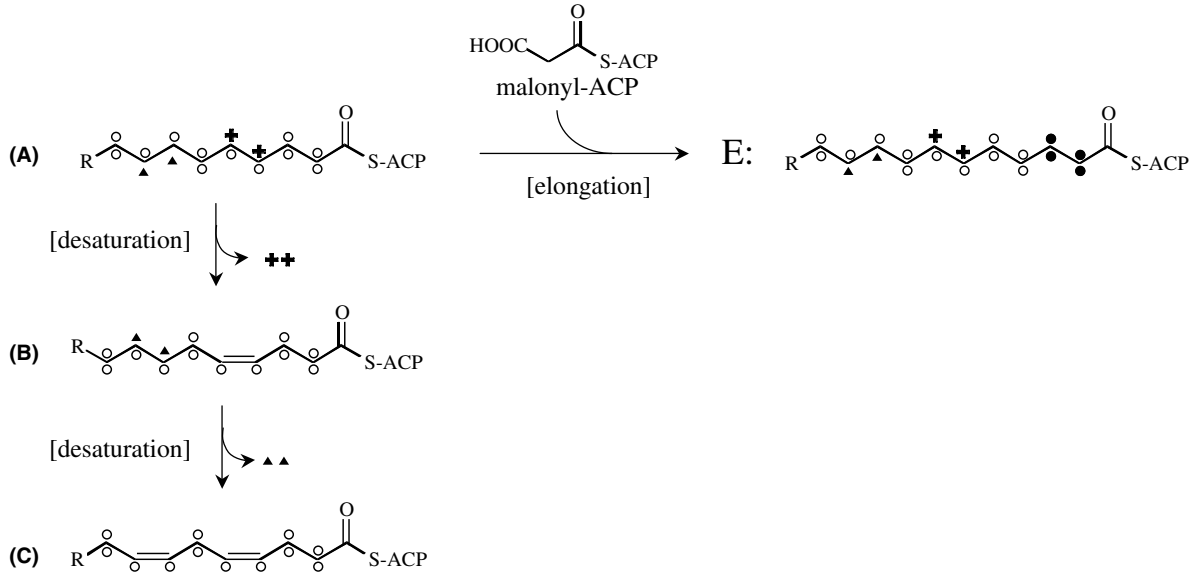

Fig. 2. A simplified model of desaturation and elongation (after Harwood and Jones, 1989; Behrouzian et al., 2001). Open-circle, cross, triangle and filled-circle indicate non-reacted hydrogens during desaturation and elongation, hydrogen removed from A to form B, hydrogen removed from B to form C, and hydrogen incorporated into E during elongation, respectively.

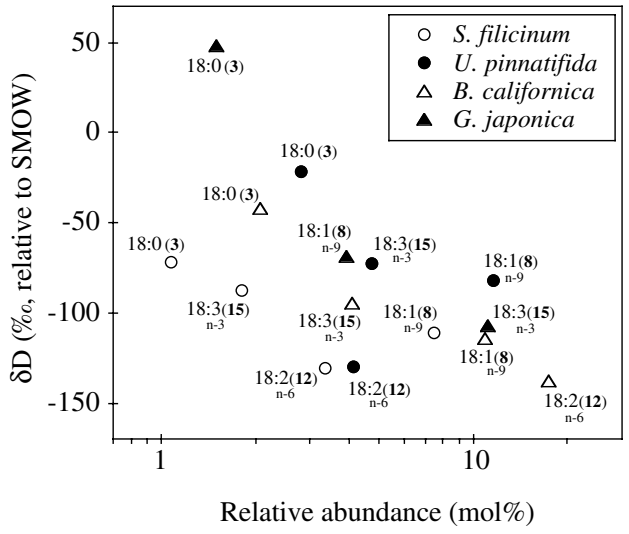

Fig. 3. Relationship between δD values and relative abundance (mol%) for 18:0 (**3**), 18:1($n-9$) (**8**), 18:2($n-6$) (**12**) and 18:3($n-3$) (**15**).

riched in D relative to high abundant fatty acids. The significant D-enrichment of the low abundant fatty acids could be due to high flux into their desaturates. Similarly, in the case of desaturation of 20:4($n-3$) (**21**) to form 20:5($n-3$) (**22**), a large δD difference between molecules is found in *U. pinnatifida* (~150‰) and *B. californica* (~80‰). This suggests that the two hydrogens remaining on residual 20:4($n-3$) (**21**) should be extremely enriched in D by isotope effect during desaturation with high flux into 20:5($n-3$) (**22**). The abundance of 20:4($n-3$) (**21**) relative to 20:5($n-3$) (**22**) is less than 10% for both algae. The distinct D-enrichments may indicate a different flux during desaturation between algae. This explanation can be also applied to explain δD difference between 20:0 (**4**) and eicosenoic acid [20:1($n-11$)] (**9**), and between 22:0 (**5**) and docosenoic acid [22:1($n-13$)] (**10**).

For all macroalgae, no substantial difference is found in δD values between 14:0 (**1**) and 16:0 (**2**). This is because 16:0 (**2**) is abundant enough to buffer the isotopic change in D-enrichment during little flux of its desaturation, which indicates that the mean δD value of four hydrogens incorporated during elongation of 14:0 (**1**) to form 16:0 (**2**) is similar with that of hydrogens in 14:0 (**1**). This also suggests that net hydrogen isotopic fractionation during elongation should be quite small or zero. Generally, it is expected that δD value of the hydrogen incorporated during hydrogenation should be extremely depleted in D, as similar to that the hydrogen incorporated to form phytol from geranylgeranyl pyrophosphate in higher plants is estimated to be −600‰ (Chikaraishi et al., 2004). However, the elongation is more complex biochemical processes containing condensation, reduction, dehydration and a second reduction (e.g., Harwood, 1994; Post-Beittenmiller, 1996). In particular, these processes include keto–enol tautomerization accompanied with hydrogen-exchange reaction, which causes a large contribution of hydrogen derived from cell water (Sessions et al., 2002; Chikaraishi et al., 2004). In the case of marine algae, δD value of cell water would be equal to that of seawater (δD ~0‰), being more enriched in D compared with the hydrogen incorporated during hydrogenation (Chikaraishi et al., 2004). In addition, even though 14:0 (**1**) is biosynthesized by continuous elongations, each elongation is composed of the same biochemical processes (Harwood, 1994; Post-Beittenmiller, 1996). Therefore, it is likely that the mean δD value of the four hydrogens incorporated during elongation to form 16:0 (**2**) is similar to that of hydrogens in 14:0 (**1**). On the other hand, a large δD difference (up to 183‰) between 16:0 (**2**) and 18:0 (**3**) is observed in all macroalgae. If the observed δD difference might be due to D-enrichment during elongation of 16:0 (**2**) to form 18:0 (**3**), the incorporated hydrogen should be extremely enriched in D. However, such an extreme D-enrichment is significantly different from isotope signature during elongations until 16:0 (**2**). Probably, 18:0 (**3**) abundance with its high flux for desaturation suggests that a large δD difference (up to 183‰) between 16:0 (**2**) and 18:0 (**3**) is not due to unusual extreme D-enrichment of the hydrogen incorporated during elongation of 16:0 (**2**) to form 18:0 (**3**), but rather due to D-enrichment associated with high flux for the following desaturation processes. 20:0 (**4**) and 22:0 (**5**) as well as their mono-desaturates such as 20:1($n-11$) (**9**) and 22:1($n-13$) (**10**) are also enriched in D than 14:0 (**1**) and 16:0 (**2**). This suggests that they are biosynthesized by using D-enriched 18:0 (**3**) as a primer.

Thus, wide δD variation (up to 183‰) of fatty acids within a single species is probably explained by the hydrogen isotopic fractionation during desaturation, which is much larger than net hydrogen isotopic fractionation during elongation in the complex network of polyunsaturated fatty acid biosynthesis.

3. Conclusions

Compound-specific hydrogen isotopic compositions of saturated, mono-unsaturated and polyunsaturated fatty acids are determined for natural marine macroalgae. δD values of individual fatty acids from four macroalgae exhibit a wide variation ranging from −189‰ to +48‰ (Table 1), which is explained by the hydrogen isotopic fractionation during desaturation. The isotopic effect during desaturation should be much larger than that during elongation in the complex network of polyunsaturated fatty acid biosynthesis. A large hydrogen isotopic fractionation during desaturation may cause D-enrichment in the remaining hydrogen of the residual fatty acids, which could be controlled by the relative flux into their desaturates. On the other

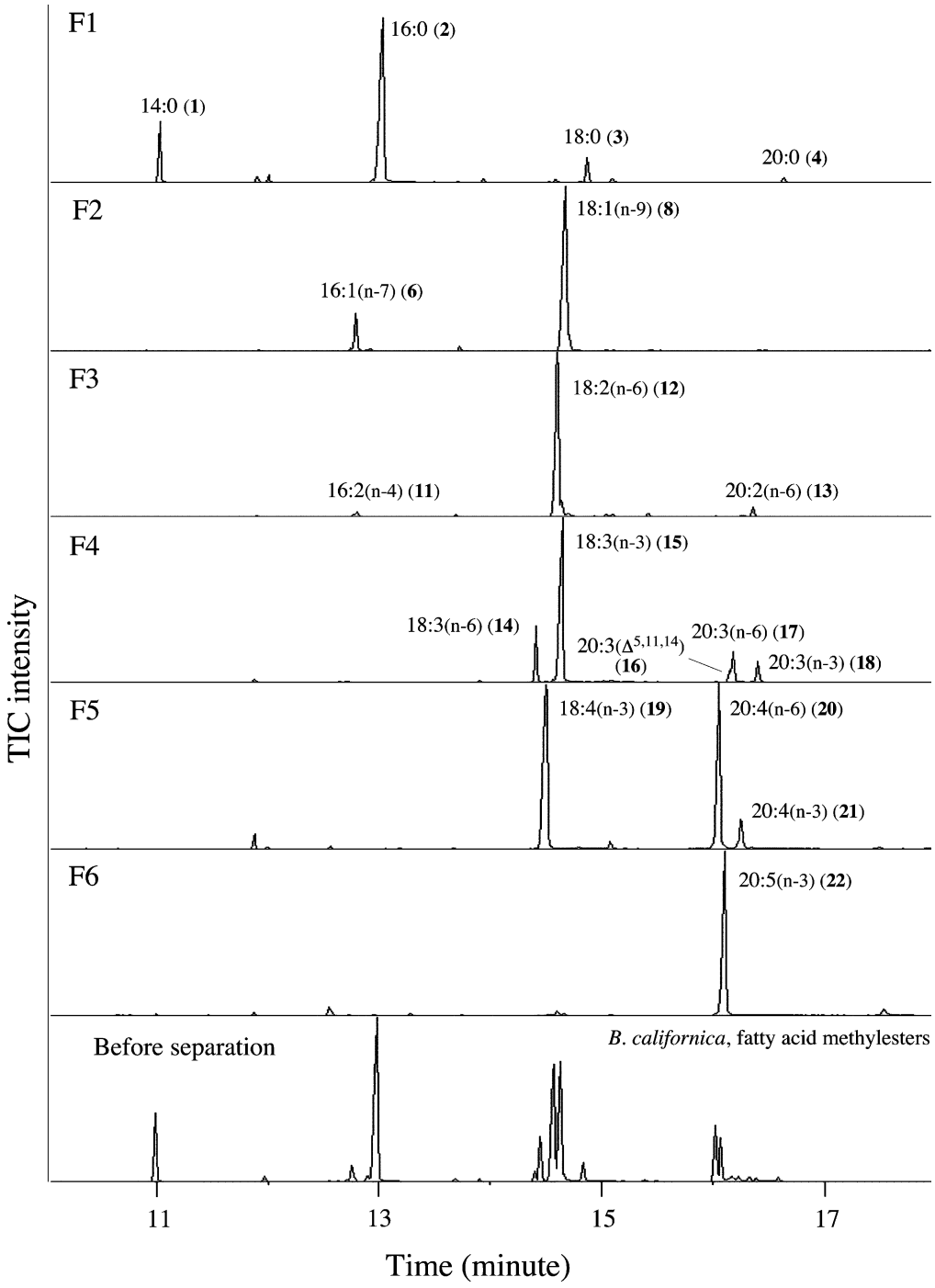

Fig. 4. Representative gas chromatograms for (*B. californica*). Fatty acid methylesters (FAMEs) were separated into six fractions using silver nitrate (10 wt%) impregnated silica gel CC, in order to measure more precise isotopic composition: F1 (saturated FAMEs); F2 (monounsaturated FAMEs); F3 (diunsaturated FAMEs); F4 (triunsaturated FAMEs); F5 (tetraunsaturated FAMEs) and F6 (pentaunsaturated FAMEs).

hand, net hydrogen isotopic fractionation during elongation should be quite small or zero. These results provide knowledge of hydrogen isotopic fractionations during desaturation and elongation associated with polyunsaturated fatty acid biosynthesis, which is required for the better understanding of hydrogen isotopic distributions of individual fatty acids in natural environment, including biological and geochemical samples. Particularly, since the hydrogen isotopic compositions of fatty acids will depend on the relative flux along the desaturation process, comparison of the δD values between different fatty acids should become a useful tool to quantify the flow of molecules through biosynthetic network.

4. Experimental

4.1. Instrumentation

Fatty acid methyl esters (FAMEs) were identified by gas chromatography/mass spectrometry (GC/MS) using an HP 6890 gas chromatograph connected to an HP MSD 5972A mass spectrometer. Location of the double bonds in mono- and poly-unsaturated fatty acids was determined by formation of dimethyl disulfide (DMDS) adducts (Leonhardt and DeVilbiss, 1985; Nichols et al., 1986; Scribe et al., 1988) and dimethyloxazoline (DMOX) derivatives (Fay and Richli, 1991; Luthria and Sprecher, 1993; Christie, 1998, 2003), respectively. The abundance of the FAMEs was quantified using external *n*-alkane standards eluted on an HP 6890 GC equipped with with a flame ionization detector (FID).

Compound-specific carbon and hydrogen isotope analyses were carried out by GC/combustion/isotope ratio MS (GC/combustion/IRMS) using a Finnigan Delta S interfaced with HP 5890 GC, and GC/pyrolysis/IRMS using a Finnigan Delta plus XL interfaced with HP 6890 GC, respectively. Combustions were performed in a microvolume ceramic tube with CuO and Pt wires at 840 °C (Hayes et al., 1990). Pyrolyses were performed in a microvolume ceramic tube with graphite at 1440 °C (Burgøyne and Hayes, 1998; Hilkert et al., 1999). $\delta^{13}C$ and δD values are given in per mil (‰) relative to PDB and SMOW, respectively. Carbon and hydrogen isotopic compositions were calibrated by coinjected internal *n*-alkane standards (Chikaraishi et al., 2004). Standard deviations of carbon and hydrogen isotope measurement were generally better than 0.5‰ (~0.3‰ in average) and 7‰ (~3‰ in average), respectively. The measured $\delta^{13}C$ and δD values of individual fatty acids were corrected by isotopic mass balance for contribution of carbon (−72.5‰) and hydrogen (−235‰) added during esterification (Chikaraishi et al., 2004).

4.2. Plant material and FAME isolation

Four marine macroalgae including two brown algae (Heterokontophyta) and two red algae (Rhodophyta) were collected from the sea about 2–4 m in depth along the seacoast near Tokyo in February 2001. The collected macroalgae were stored at −20 °C until analysis. These macroalgae were individually freeze-dried and crushed to a fine powder, with dried samples saponified with 0.5 M KOH in MeOH/H_2O (95/5, w/w) by refluxing for 4.5 h and extracted with CH_2Cl_2/MeOH (2/1, v/v) by sonication to isolate lipids. The extracted lipids were esterified using 14% BF_3/MeOH (100 °C, 1 h) to form fatty acid methyl esters (FAMEs).

The FAMEs were further separated into six fractions based on their degrees of unsaturation: F1 (saturated FAMEs); F2 (monounsaturated FAMEs); F3 (diunsaturated FAMEs); F4 (triunsaturated FAMEs); F5 (tetraunsaturated FAMEs) and F6 (pentaunsaturated FAMEs) using $AgNO_3$ (10 wt%) impregnated Si gel CC. The F1 fraction was separated from the other five fractions (F2, F3, F4, F5 and F6) by CC (4cm in length, 6mm in diameter), in which F1 was eluted with *n*-hexane/CH_2Cl_2 (9 ml, 4:1, v/v) and all other fractions were subsequently eluted by CH_2Cl_2/ethyl acetate (4 ml, 4:1, v/v). The F2 to F6 fractions were separated by the CC (6 cm in length, 6 mm in diameter), where F2 and F3 fractions eluted in the first 4 ml followed by CH_2Cl_2/EtOAc (4 ml, 49:1, v/v), respectively. Subsequently, F4 and F5 fractions were eluted by the first 4 ml followed by *n*-hexane/ethyl acetate (5 ml, 4:1, v/v), respectively. Finally, the F6 fraction was eluted with CH_2Cl_2/ethyl acetate (2 ml, 3:1, v/v) followed by *n*-hexane/EtOAc (5 ml, 4:1, v/v). Representative chromatograms before and after separation are shown in Fig. 4.

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