Res. Org. Geochem. **37**, 67 − 72 (2021)

Articles

A large fractionation of $^{13}C/^{12}C$ ratios for palmitate metabolism in plants

Yuko Takizawa [*, a] and Yoshito Chikaraishi [*, **]

(Received November 19, 2021; Accepted December 26)

Abstract

Isotopic compositions in fatty acids have widely been used in the study for tracing sources and delivery of organic materials in the earth surface and for reconstructing environments in the geological timescale. Changes in the isotopic compositions associated with the lipid degradation of organisms, however, have been poorly understood. In this study, we determined changes in the carbon and hydrogen isotopic compositions (i.e., the $\delta^{13}C$ and δD values, respectively) of palmitate (i.e., $C_{16:0}$ carboxylic acid esters), a major alkanoate in eukarya and bacteria, during the germination of plant seeds under no photosynthetic activity. During the germination, abundance of palmitate is decreased by 41 and 94 % for 5 and 14 days, which causes a considerable change in the $\delta^{13}C$ value of palmitate by 3.0 and 17.2‰, respectively. In contrast, non-substantial difference is found in the δD values of palmitate for this germination. These enrichments indicate that isotopic fractionation occurs on a carbon but not hydrogen atom of palmitate in an initial reaction of the degradation, as reaction of carbonyl carbon in palmitate with enzyme (lipase) can be proposed for a candidate mechanism. If such reaction mainly occurs on palmitate components in triacylglycerol for the germination of plant seeds, the enrichment in ^{13}C estimated is more than 250‰ for the carbonyl carbon in palmitate based on a mass balance calculation, and the isotope fractionation factor (α) accounts for 0.9086 in this reaction based on the Rayleigh fractionation model.

1. Introduction

Organic compounds have a wide diversity in the isotope ratio among samples (e.g., primary producers and their source CO_2) and a large variation in it within a sample (e.g., temporal and spatial changes) in natural environments. In general, the isotopic compositions of organic compounds are explained by two factors: the isotopic compositions of substrates (δ_{sub}) and the isotopic fractionation of reactions (ε) that are used both for producing (ε_p) and degrading (ε_d) of organic compounds in organisms and environments (e.g., Hayes, 2001), if they are quantum chemistric non-quantitative reactions in a process of metabolism (equation 1):

$$\delta \text{ of organic compound} = (\delta_{sub} + \varepsilon_p) + \varepsilon_d \quad (1)$$

For example, the ε_p is found between CO_2 (substrates) and photosynthates (products) during plant photosynthesis and the ε_d is found between amino acids (substrates) and ammonia (products) during consumer metabolism.

The isotopic compositions in lipids also have a wide range of diversity and variation, and their compositions allow us to use in the study for characterizing sources and delivery of organic compounds and their elements in biogeochemical cycles, and illustrating environments where, when, and/or how the lipids were produced (e.g., Pancost and Sinninghe Damsté 2003; Eglinton and Eglinton, 2008; Sachse et al., 2012). However, isotopic fractionation associated with lipid degradation found within an organism, between organisms, and in environments (e.g., water column and sediments) has been poorly understood so far. Indeed, we have long been simply thought that isotopic fractionation associated with the degradation of large carbon numbered organic compounds such as lipids is negligible (e.g., Huang et al., 1997; Mazeas et al., 2002; Chikaraishi and Naraoka, 2006). For the example of carbon, if there is a significant isotopic fractionation on a key reacting carbon atom ($\Delta\delta^{13}C_{key}$) in a compound, the $\Delta\delta^{13}C_{key}$ value is much diluted with no isotopic fractionation on numbers of non-reacting carbon atoms ($\Delta\delta^{13}C_{other}$) within the same compound (equations 2 and 3).

$$n \times \Delta\delta^{13}C_m = 1 \times \Delta\delta^{13}C_{key} + (n-1) \times \Delta\delta^{13}C_{other} \quad (2)$$

where $\Delta\delta^{13}C_m$ indicates change in the measured $\delta^{13}C$ value of compounds between before and after reaction, and n indicates the number of carbon atom in the com-

[*] Institute of Low Temperature Science, Hokkaido University, N19W8, Kita-ku, Sapporo, Japan, 060-0819
[**] The Japan Agency for Marine-Earth Science and Technology, 2-15, Natsushima-cho, Yokosuka, Japan, 237-0061
[a] Corresponding author. e-mail: takizaway@lowtem.hokudai.ac.jp

pound. Because the $\Delta\delta^{13}C_{other}$ value is zero ($\Delta\delta^{13}C_{other} = 0$) in many cases, the equation (2) is given by:

$$\Delta\delta^{13}C_m = \frac{1}{n} \times \Delta\delta^{13}C_{key} \quad (3)$$

meaning that, for example, the $\Delta\delta^{13}C_m$ value of palmitate (n=16) is considerably small, 0.6‰, even if the $\Delta\delta^{13}C_{key}$ value is 10.0‰. As similar to carbon, $\Delta\delta D_{key}$ value is much diluted with no isotopic fractionation on numbers of non-reacting hydrogen atoms ($\Delta\delta D_{other}$) within the same compound (equation 4).

$$\Delta\delta D_m = \frac{1}{n} \times \Delta\delta D_{key} \quad (4)$$

where $\Delta\delta D_m$ indicates change in the measured δD value of compounds between before and after reaction, and n indicates the number of hydrogen atoms in the compound. The $\Delta\delta D_m$ value of palmitate (n=32) is considerably small, 3.1‰, even if the $\Delta\delta D_{key}$ value is 100‰. These $\Delta\delta_m$ values for the carbon (0.6‰) and hydrogen (3.1‰) are as small as analytical precision of molecular isotope analysis (e.g., Brand, 1996; Hilkert et al. 1999; 0.3-0.8 and 3-8‰, respectively).

In this study, we designed laboratory pot experiments of plant seed germination under no photosynthetic activity, to detect isotopic fractionations ($\Delta\delta^{13}C_m$ and $\Delta\delta D_m$) of palmitate, as a dominant lipid compound, during lipid degradation in plants. As a represent of plant seeds that contain considerable abundance of storage lipids, the sesame *Sesamum indicum* were used for the pot experiments. Because they degrade storage lipids as almost solo energy and substrate sources for germination, we hypothesized that when a large proportion of lipids in seeds is degraded during experiment, the $\Delta\delta^{13}C_m$ and/or $\Delta\delta D_m$ values will be significantly large enough to measure even much diluted. Based on the results, we will clarify whether or not there are significant isotopic fractionation during lipid degradation, and discuss potential mechanism to explain the $\Delta\delta^{13}C$ and $\Delta\delta D$ values observed.

2. Materials and Methods

2.1. Experimental procedure

In this study, we conducted two experiments of plant seed germination. Seeds of the sesame *Sesamum indicum* were purchased from plant seed stores and an aliquot of seeds were germinated in a pot under dark condition for 5 and 14 days. Remaining seeds and developed sprouts were stored at −20°C until analysis.

Palmitate was extracted from seeds and sprouts according to a modified procedure in Chikaraishi and Naraoka (2005). In brief, the frozen sample was saponified with KOH in CH_3OH/H_2O (95/5, w/w) to hydrolyze ester-bonds. Neutral lipids were removed with *n*-hexane/dichloromethane (4/1, v/v), and subsequently acidic lipids (including palmitate) were extracted with *n*-hexane/dichloromethane (4/1, v/v) after addition of 12M HCl (to pH<1). The acidic lipids were esterified with CH_3OH to form methylester derivatives.

2.2. Measurement for the $\delta^{13}C$ and δD values with GC-IRMS

The $\delta^{13}C$ and δD values of palmitate derivatives were measured with a gas chromatograph-isotope ratio mass spectrometer (GC-IRMS), and the following two instrument conditions (i) and (ii) were used (but independently for each experiment) for 5 and 14 days experiments, respectively.

(i) An Agilent Technologies 7890A GC coupled to a Thermo Fisher Scientific $Delta_{plus}XP$ IRMS with a GC-Isolink interface, with a combustion temperature of 1020° C for carbon and a pyrolysis temperature of 1450° C for hydrogen (e.g., Chikaraishi et al., 2012). A programmable temperature vaporizing (PTV) injector (GERSTEL) was used for injection of palmitate derivative into an HP-5ms capillary column (30 m; i.d. 0.25 mm; film thickness 0.25μm; Agilent Technologies).

(ii) An Agilent Technologies 7890B GC coupled to a Thermo Fisher Scientific DeltaV IRMS with a GC-C III interface, with a combustion temperature of 950° C for carbon and a pyrolysis temperature of 1440° C for hydrogen. A PTV injector (GERSTEL) was also used for injection of palmitate derivative into an HP-5ms capillary column (60 m; i.d. 0.25 mm; film thickness 0.10μm; Agilent Technologies).

Two pulses of reference CO_2 or H_2 gas were discharged into the IRMS at the commencement and completion of each chromatography run, for both reference mixtures and samples. The isotopic compositions are expressed relative to Vienna Peedee Belemnite (VPDB) and Vienna Standard Mean Ocean Water (VSMOW), on scales normalized to the known δ values of the reference *n*-alkanes (Chikaraishi and Naraoka, 2007). The standard deviation of measurements for the reference *n*-alkanes was better than 1.2‰ for carbon and 3‰ for hydrogen, respectively, in the instrument conditions (i) and better than 0.3‰ for carbon and 6‰ for hydrogen, respectively, in the instrument condition (ii).

3. Results and Discussion

3.1. $\delta^{13}C$ and δD of palmitate in sesame seeds and sprouts

The molecular abundance of palmitate is decreased

by 41% from seeds (t=0) to sprouts (t=5), and by 94% from seeds (t=0) to sprouts (t=14) for the germination experiments (Figure 1a). The isotopic compositions of palmitate are increased considerably from −34.0‰ to −31.1‰ and from −35.6‰ to −18.3‰ for carbon but negligibly from −189‰ to −191‰ and from −174‰ to −172‰ for hydrogen in the germination experiments (Table 1, Figure 1b,c). Comparing to the standard deviation of isotope analysis, we recognize the existence of significant $\Delta\delta^{13}C$ but insignificant $\Delta\delta D$ values of palmitate during the plant seed germination, which is attributable to a large and a little isotopic fractionation, respectively, associated with the degradation of storage lipids. Thus, although negligible isotopic fractionation has long been thought so far, the isotopic fractionation on the single carbon atom is considerably large (i.e., $\Delta\delta^{13}C_{key}$ >10‰ in eq.3) and provide that the $\Delta\delta^{13}C_m$ values enough to measure even much diluted with numbers of the carbon atoms (i.e., n=16 in eq.3) that have no isotopic fractionation. On the other hand, isotopic fractionation on hydrogen atoms is considerably small or almost zero (i.e., $\Delta\delta D_{key}$ = ~0‰ in eq.4) and further diluted with numbers of hydrogen atoms (i.e., n=32 in eq.4) that has no isotopic fractionation.

Table 1. The $\delta^{13}C$ and δD values and relative area of palmitate for individual samples.

Terms*	Days	Carbon (‰)		Hydrogen (‰)		Relative area
		δ	Δ**	δ	Δ**	
#1	0	-34.0		-189		1.00
#1	5	-31.1	3.0	-191	-2	0.59
#2	0	-35.6		-174		1.00
#2	14	-18.3	17.2	-172	2	0.06

* Terms mean 'experimntal term in this study'.
** Δ means the substraction of t=x from t=0.

3.2. Possible mechanism for the enrichment in ^{13}C

It is known that plant seeds store triacylglycerols (TAGs: a TAG consists of three alkanoates esterified to a glycerol back-bone) in organelles that are frequently called 'oil bodies' (Figure 2). This organelle has a function to provide sources of carbon and energy for germination of plant seeds (e.g., Buchanan et al., 2000). TAGs are hydrolyzed sequentially with enzyme, lipase, to alkanoate and glycerol units, and these units are incorporated into the central pathway as energy and substrate resources to produce other components such as carbohydrates and amino acids in plant growth. According to the knowledge explained above and results found in this study, we simply hypothesize that 'key' process of the isotopic fractionation observed is the connection of lipase to a carbonyl carbon atom on alkanoate components in TAGs. The following processes are considered to explain the isotopic fractionation observed (Figure 3):

(1) the enzyme (lipase) preferentially connects to ^{12}C carbonyl carbon of alkanoate components in the first step of hydrolyzation of TAGs, leaving behind the enriched ^{13}C on alkanoate components in the residual pool of TAGs;
(2) the hydrolyzed products (i.e., acyl-CoA) are subsequently used as metabolic intermediates (e.g., acetyl-CoA and succinate) and energy sources;
(3) the enrichment in ^{13}C can be found in alkanoates that are extracted by saponification of the residual pool of TAGs in sprouts (i.e., t=5, and t=14, in this study).

If it is case, the $\Delta\delta^{13}C_{key}$ values calculated by mass balance with the equation (2) is 48 ± 27‰ and 292 ± 8.5‰ (t=5 and t=14, respectively) for the carbonyl carbon of palmitate components in the residual pool of TAGs.

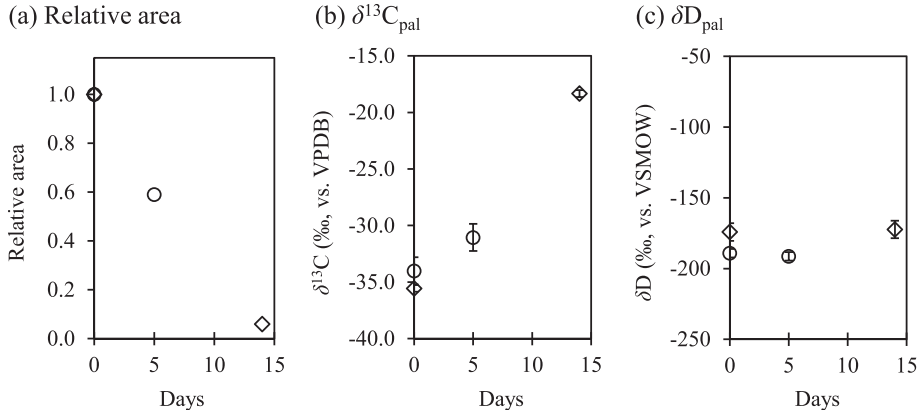

Figure 1. (a) Relative abundance, (b) the $\delta^{13}C$ values, and (c) the δD values of palmitate in seed (0 day) and sprouts (5 and 14 days) for the experiments. Experimental terms #1 and #2 are shown as open circle and open diamond, respectively. Error bars in (b) and (c) indicate the standard deviation (1σ) of replication analysis of standard materials.

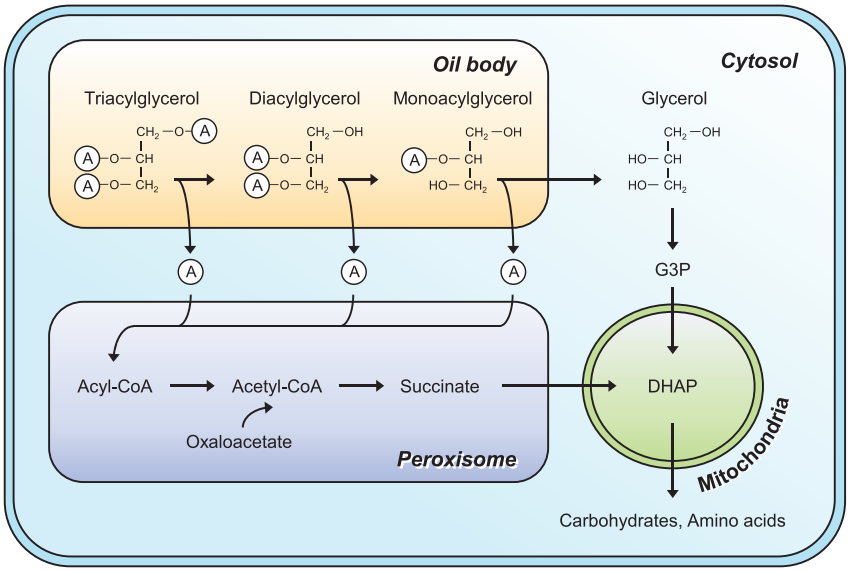

Figure 2. Schematic illustration for degradation of triacylglycerols (TAGs) stored in oil bodies. The TAGs are hydrolyzed sequentially with lipases to alkanoates (A) and glycerol. The glycerol is converted to dihydroxyacetone phosphate (DHAP) with glycerol kinase *via* Gly-3-phosphate (G3P) dehydrogenase. The alkanoates are transported into the peroxisome, where they are activated as acyl-CoAs, and enter the *β*-oxidation spiral. Acetyl-CoA condenses with oxaloacetate to form succinate. The succinate and DHAP are converted to other components such as carbohydrates and amino acids (After Quttier and Eastmond, 2009; Barros et al., 2010).

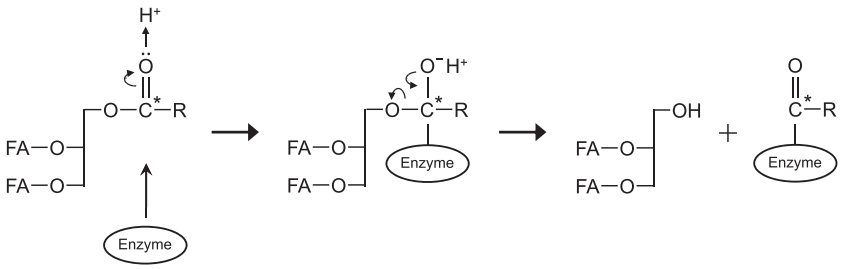

Figure 3. Possible key reaction to explain carbon isotopic fractionation during germination experiments. Asterisk (*) shows the key reaction that carbon atom reacted with enzyme in the first step of hydrolyzation during germination.

3.3. Isotopic fractionation factor

To understand isotopic fractionation during plant seed germination, we apply observed results to the Rayleigh fractionation model (Lord Rayleigh, 1986) that change in the $\delta^{13}C$ value of palmitate components in the residual pool of TAGs is given by:

$$\delta^{13}C_{pal\_t} = (1000 + \delta^{13}C_{pal\_o}) \times F^{(\alpha-1)} - 1000 \qquad (5)$$

where $\delta^{13}C_{pal\_o}$ and $\delta^{13}C_{pal\_t}$ represent the $\delta^{13}C$ values of palmitate extracted from plant seeds (t=0) and sprouts (t=x), respectively; F ($0<F<1$) represents the proportion of palmitate components in the residual pool of TAGs against enzymatic hydrolyzation (i.e., [1–F] represents the proportion of palmitate components hydrolyzed); and α represents the isotopic fractionation factor of the enzymatic hydrolyzation. Rearrangement of the equation (5) leads to equation (6):

$$\ln[(1000 + \delta^{13}C_{pal\_t})/(1000 + \delta^{13}C_{pal\_o})] = (\alpha-1) \times \ln F \qquad (6)$$

The equation (6) means that '$\ln[(1000 + \delta^{13}C_{pal\_t})/(1000 + \delta^{13}C_{pal\_o})]$' shows a strong negative linear correlation with '$\ln F$', and that '$(\alpha-1)$' shows the slope of the linear regression line with the intercept being zero, as y= ax. Applying the observed data to equation (6), we found the strong negative linear correlation

(R^2=0.9975) with slope being −0.0914 (Figure 4a).

Based on the results of this study, change in the $\delta^{13}C_{pal}$ value found in plant seeds germination certainly follows the Rayleigh fractionation model. From the value of (α−1), isotope fractionation factor (α) calculated is 0.9086. Thus, the α lower than 1.0 (i.e., α=0.9086) clearly indicates that the ^{13}C-enrichment of palmitate components ($\Delta\delta^{13}C_{pal\_t}$) increases inversely with the F as a solo factor, implying that the hydrolyzation preferentially reacts with ^{12}C palmitate components in TAGs during the lipid degradation. According to α=0.9086 observed, we can calculate 20‰ and 200‰ for the $\Delta\delta$ ^{13}C values of carbonyl carbon on palmitate components in the remaining pool of TAGs, and 1.25‰ and 12.5‰, respectively, for those of whole palmitate components, if TAGs are degraded by 19% and 86%, respectively, with no isotopic fractionation being observed on the alkyl chain (Figure 4b).

3.4. Notes for application of the isotopic fractionation

The $\Delta\delta^{13}C$ values of carbonyl carbon can directly explain the degradation flux of palmitate components, because seeds were germinated in a pot under dark condition (i.e., no input of palmitate from *de novo* synthesis) in this study. However, in natural environments, both plants and animals can *de novo* synthesize and degrade palmitate, and such palmitate are incorporated directly *via* salvage pathways into animals that feed on plant and other animals (e.g., Kitatani et al., 2008). In such cases, the $\Delta\delta^{13}C$ values will be applicable to evaluate the balance between *de novo* production and degradation integrated in all processes that the palmitate experienced in both plant and animal in environments. For example, enrichment in ^{13}C by approximately 8‰ for palmitate from plant leaves to soils was reported in Chikaraishi and Naraoka (2006). Also, decrease in molecular abundance by approximately 80% for palmitate during litterbag experiments was reported in Nyugen Tu et al., (2017). If we roughly apply α=0.9086 to the evaluation of the integrated balance between *de novo* production and degradation on the results in these previous studies, the ^{13}C-enrichment in the former study accounts for that palmitate is degraded by 75% from leaves to soils, and the abundance decrease in the latter study accounts for that palmitate is enriched in ^{13}C by 10‰ during litterbag experiments. Although we must evaluate the other major factors that affect to the $\Delta\delta^{13}C$ values in natural environments (e.g., temporal variation, physiological condition, etc.), α observed in this study can supply a likely explanation for the changes in the $\delta^{13}C$ value and molecular abundance of palmitate found in these previous studies.

4. Conclusion

In this study, we determined significant enrichment in ^{13}C on palmitate, as a dominant lipid compound, during significant lipid degradation in plant seeds, which are explained by followings:

(1) Preferential bounding of enzyme (lipase) to ^{12}C carbonyl carbon of alkanoate components in TAGs is the key process controlling for the significant enrichment in ^{13}C on palmitate;
(2) Based on the Rayleigh fractionation model, the isotope fractionation factor (α) accounts for 0.9086 in the enzymatic hydrolyzation of TAGs.

This α=0.9086 implies that a considerable enrichment in ^{13}C more than 100‰ can be observed for the carbonyl carbon of palmitate components, as, in this

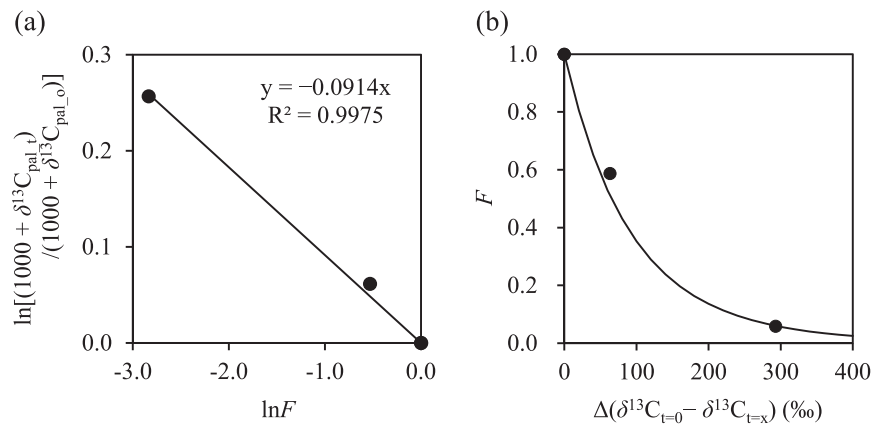

Figure 4. (a) The isotopic isotopic fractionation factor (α) for lipid degradation during plant germination. The α value was estimated data observed in the present study with the Rayleigh fractionation model that indicates a negative linear correlation between $\ln[(1000 + \delta^{13}C_{pal\_t})/(1000 + \delta^{13}C_{pal\_o})]$ and $\ln F$ with slope of (α−1). (b) The relationship between $\Delta(\delta^{13}C_{t=0} - \delta^{13}C_{t=x})$ and metabolic flux (F) found in this study. The solid curve line indicates Δ estimated by the Rayleigh fractionation model with α=0.9086

study, more than 250‰ for the $\Delta\delta^{13}C$ values of the carbonyl carbon in the remaining pool of TAGs is observed when TAGs are degraded by 94% during germination for 14 days.

To proof the enrichment in ^{13}C observed, we expect that development of position-specific isotope analysis for carbonyl carbon in palmitate will be highly useful, as a method directly for measuring the enrichment. This development will further contribute for better understanding the quantitative evaluation of lipid degradation found in organisms and environments and for enhancing the utilization of lipid as biomarkers for studies in organic geochemistry.

Acknowledgment

We thank to Prof. Ken Sawada and Dr. Yoshinori Takano for giving constructive comments during review process of this manuscript. This work was supported by JSPS KAKENHI Grant Number JP 16J01704 and 20K14590 to Y. Takizawa, Leadership Research Grant from Institute of Low Temperature Science, Hokkaido University (FY2019) to Y. Takizawa, Grant-in-Aid for Scientific Research of the JSPS No. 17H02988 and 20H00185 to Y. Chikaraishi, and funds from Silicone Industry Association of Japan to Y. Chikaraishi.

References

Barros M., Fleuri L. F., and Macedo G. A. (2010) Seed lipases: Sources, applications and properties - A review. *Braz. J. Chem. Eng.* 27, 12-29.

Brand W. A. (1996) High precision isotope ratio monitoring techniques in mass spectrometry. *J. Mass Spectrom.* 31, 225-235.

Buchanan B. B., Gruissem W., and Jones R. L. (2000) Biochemistry and Molecular Biology of Plants, *Am. Soc. Plant Physiol.* Rockville, Maryland, USA.

Chikaraishi Y. and Naraoka H. (2005), $\delta^{13}C$ and δD identification of sources of lipid biomarkers in sediments of Lake Haruna (Japan). *Geochim. Cosmochim. Acta.* 69, 3285-3297.

Chikaraishi Y. and Naraoka H. (2006) Carbon and hydrogen isotope variation of plant biomarkers in a plant-soil system. *Chem. Geol.* 231, 190-202.

Chikaraishi Y. and Naraoka H. (2007) $\delta^{13}C$ and δD relationships among three *n*-alkyl compound classes (*n*-alkanoic acid, *n*-alkane and *n*-alkanol) of terrestrial higher plants. *Org. Geochem*. 38, 198-215.

Chikaraishi Y. Kaneko M. and Ohkouchi N. (2012) Stable hydrogen and carbon isotopic compositions of long-chain (C_{21}-C_{33}) *n*-alkanes and *n*-alkenes in insects. *Geochim. Cosmochim. Acta,* 95, 53-62.

Eglinton T. I. and Eglinton G. (2008) Molecular proxies for paleoclimatology. *Earth Planet. Sci. Lett.* 275, 1-16.

Hayes J. M. (2001) Fractionation of carbon and hydrogen isotopes in biosynthetic processes. In: Valley, J.W., Cole, D.R. (Eds.), *Reviews in Mineralogy and Geochemistry 43, Stable Isotope Geochemistry.* The Mineralogical Society of America, Washington, pp. 225-277.

Hilkert A. W., Douthitt C. B., Schlüter H. J. and Brand W. A. (1999) Isotope ratio monitoring gas chromatography/mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 13, 1226-1230.

Huang, Y., Eglinton, G., Ineson, P., Latter, P. M., Bol, R., Harkness, D. D. (1997) Absence of carbon isotope fractionation of individual *n*-alkanes in a 23-year field decomposition experiment with *Calluna vulgaris*. *Org. Geochem.* 26, 497-501.

Kitatani, K., Idkowiak-Baldys, J. and Hannun, Y.A. (2008) The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell. Signal.* 20, 1010-1018.

Mazeas, L., Budzinski, H., Raymond, N. (2002) Absence of stable carbon isotope fractionation of saturated and polycyclic aromatic hydrocarbons during aerobic bacterial biodegradation. *Org. Geochem.* 33, 1259-1272.

Nguyen Tu T. T., Egasse C., Anquetil C., Zanetti F., Zeller B., Huon S., Derenne S. (2017) Leaf lipid degradation in soils and surface sediments: A litterbag experiment. *Org. Geochem.* 104, 35-41.

Pancost R. D. and J. S. Sinninghe Damsté (2003) Carbon isotopic compositions of prokaryotic lipids as tracers of carbon cycling in diverse settings. *Chem. Geol.* , 195, 29-58.

Quttier A. L. and Eastmond P. J. (2009) Storage oil hydrolysis during early seedling growth. *Plant Physiol. Bioch.* 47, 485-490.

Lord Rayleigh Sec. R.S. (1896) L. Theoretical considerations respecting the separation of gases by diffusion and similar processes, *Philosoph. Mag. J. Sci.* 42, 493-498.

Sachse D., Billault I., Bowen G. J., Chikaraishi Y., Dawson T. D., Feakins S. J., Freeman K. H., Magill C. R., McInerney F. A., van der Meer M. T. J., Polissar P., Robins R. J., Sachs J. P., Schmidt H.-L., Sessions A. L., White J. W. C., West J. B. and Kahmen A. (2012) Molecular paleohydrology: interpreting the hydrogen-isotopic composition of lipid biomarkers from photosynthesizing organisms. *Annu. Rev. Earth Planet. Sci.* 40, 221-249.