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Correlation analysis between fatty acid compositions of zooplankter individuals, fed on different phytoplankton species by means of pyrolysis–gas chromatography combined with on-line methylation

Yasuyuki Ishida[a], Hiroaki Yokoi[a], Shinichi Isomura[a], Hajime Ohtani[a], Shin Tsuge[a,*], Tatsuki Sekino[b], Masami Nakanishi[b], Takashi Kimoto[c]

[a]*Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan*

[b]*Center for Ecological Research, Kyoto University, Shimosakamoto, Otsu 520-0105, Japan*

[c]*Research Institute of Oceano-Chemistry, Funabashi-cho, Tenouji-ku, Osaka 543-0024, Japan*

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Abstract

Pyrolysis–gas chromatography (Py–GC) combined with on-line methylation was applied to a correlation analysis between the distributions of fatty acid components in the lipids of zooplankter individuals and those of ingested algae using principal component analysis (PCA). Py–GC in the presence of organic alkali, tetramethylammonium hydroxide (TMAH), was used to estimate the apparent distributions of fatty acid components contained in a single individual zooplankter weighing several tens of micrograms and a small sample size of ingested algae samples in the order of 10 μg. The observed fatty acid compositions were used as a database for the PCA in order to discriminate the zooplankton and ingested algae samples. The result obtained indicated that the fatty acid compositions of zooplankton individuals used in this work were significantly reflected in those of their ingested food in spite of some contribution from isomerization and/or elongation of fatty acid components during digestion of the ingested algae phytoplankton in living zooplankters. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Zooplankter; Pyrolysis; Methylation; Fatty acids

1. Introduction

The fatty acid composition of a lipid component contained in zooplankton is affected not only by its environmental conditions, such as seasonal changes, geographic origin and sampling depth, but also by the species of phytoplankton ingested as their food. Therefore, information about lipid metabolism or life history of zooplankton can be obtained by estimating a relationship between fatty acid compositions of zooplankton and their food. In recent years, it is of interest to determine the fatty acid composition of a single individual zooplankter in order to consider its metabolism or life history in terms of inter-individual differences. Also, in the field of geochemical and environmental chemistry, it has often been requested to evaluate the correlation among the fatty acid compositions of very small amounts of organic

*Corresponding author.

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matter, a single zooplankter or a few micrograms of phytoplankton sample, in order to obtain detailed information about the food web or the cycling of carbon within a very limited area in a marine and/or lake environment.

In general, the lipid components contained in zoo- and phytoplankton samples have been determined by use of pretreatment, such as solvent extraction and saponification, followed by various kinds of chromatography [1–3]. However, these techniques cannot be applied to the lipid characterization of a single zooplankter or micro amounts of phytoplankton samples because of the requirement for fairly large sample sizes, in the order of 10 mg, for the extraction procedure.

On the other hand, analytical pyrolysis methods such as pyrolysis–gas chromatography (Py–GC), Py–GC–mass spectrometry (Py–GC–MS) or Py–MS have been widely used in the fields of microbiology [4], organic geochemistry [5] and environmental chemistry [6]. Particularly, Py–MS combined with multivariate analysis has been used to characterize organic matter in the marine environment, such as aquatic plants, zooplankton, microorganisms and suspended matter in the ocean [7,8]. Although this technique provided a fairly sensitive method to evaluate the organic matter by use of minute amounts of samples in the order of 10 μg, it is difficult to yield the accurate chemical composition of lipid components because of variations in response factors for different compound classes.

Recently, pyrolysis in the presence of an organic alkali such as tetramethylammonium hydroxide [$(CH_3)_4NOH$] (TMAH) enabled the highly sensitive detection of the constituents of various polyester samples as their methyl derivatives [9,10]. This technique allowed detailed structural information to be attained not only on synthetic polymers but also on various biopolymers such as humic substances, microorganisms and lignin by converting polar products to less polar derivatives which are more amenable to chromatographic separation [11–14]. The authors have reported a highly sensitive method for detection of the fatty acid components contained in a single individual zooplankter with less than 4.5% of the relative standard deviation using this technique [15,16]. In these reports, the observed fatty acid composition of a single zooplankter sample was successfully used as the database for principal component analysis (PCA) in order to discriminate zooplankter individuals because of its good reproducibility although highly polyunsaturated fatty acid components were strongly depressed owing to thermal degradation and/or isomerization occurring to some extent [15]. In this work, Py–GC, in the presence of TMAH, was applied to the correlation analysis between the distributions of fatty acid components in the lipids of zooplankter individuals and ingested algae phytoplankton using PCA based on the fatty acid distributions observed in their pyrograms.

2. Experimental

2.1. Materials

Daphnia galeata samples were used as zooplankter samples in this work because *Daphnia* species was the most typical one commonly observed in most lake and marsh water. Fifteen plankton samples were cultured by using one of three algae species, *Chlamydomonas* sp., *Chlorella* sp. and *Scenedesmus* sp., as their food under the same physiological conditions in the laboratory. The food concentration was set at 1×10^6 cells per ml for *Chlamydomonas*, and 5×10^6 cells per ml for *Chlorella* and *Scenedesmus*. The dry weight of each zooplankter sample ranged from 37 to 62 μg.

A water solution (25 wt.%) of tetramethylammonium hydroxide (TMAH) supplied by Tama Chemicals was used as the reagent.

2.2. Conditions for Py–GC

The procedure for Py–GC combined with on-line methylation is basically the same as was described in our previous papers [15,16]. A vertical microfurnace pyrolyzer (Yanaco GP1018) was directly attached to a gas chromatograph (HP 5890) equipped with a flame ionization detector (FID). A single dried plankter or about 100 μg of phytoplankton sample was weighed using a micro-balance into a small platinum cup. After 2 μl of the TMAH solution was added to the same sample cup, it was dropped into the heated center of the pyrolyzer under the flow of

helium carrier gas. The optimum pyrolysis temperature of 400°C to attain the highest yield of fatty acid methyl esters was empirically determined after examining various temperatures between 300 and 600°C. A fused-silica capillary column (Hewlett Packard Innowax, 25 m×0.2 mm I.D.) coated with polyethyleneglycol (0.33-μm film thickness), immobilized by chemical crosslinking, was used for GC. The 50-ml/min carrier gas flow-rate at the pyrolyzer was reduced to 1.0 ml/min at the capillary column by means of a splitter. The column temperature was initially set at 50°C and then programmed up to 240°C at a rate of 5°C/min. All the phytoplankton samples were analysed with three repetitions. Identification of the peaks on the pyrograms was carried out mainly using a GC–MS system (JEOL Automass 150) with an electron impact ionization (70 eV) source to which the pyrolyzer was directly attached. The resulting data were processed by use of PCA as described by Mitsui et al. [17] in order to evaluate the relationship between zooplankton samples and their food by considering all the fatty acid components commonly observed on the pyrograms.

3. Results and discussion

Fig. 1 shows the typical pyrograms obtained from ca. 100 μg of *Chlamydomonas* (a) and *Daphnia* individuals weighing about 11 μg cultured with *Chlamydomonas* (b) at 400°C in the presence of TMAH. Although no characteristic peak was observed in the pyrograms obtained without addition of TMAH [15], a series of sharp peaks from 1 to 10 was observed on both pyrograms obtained in the presence of TMAH (Fig. 1a and b) after the elution of trimethylamine and methanol formed from the

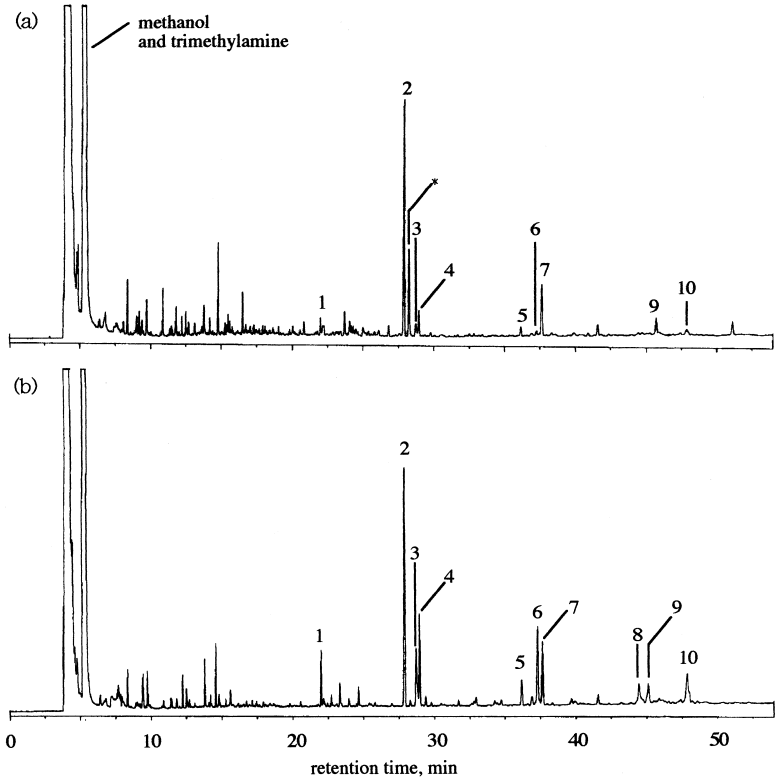

Fig. 1. Typical pyrograms of plankton samples at 400°C in the presence of TMAH. (a) Ca. 100 μg of *Chlamydomonas* sample. (b) A single *Daphnia* sample cultured with *Chlamydomonas*. Peak numbers correspond to those listed in Table 1. * This peak refers to one of the pyrolytic components of cellulose.

Table 1
Identification of the characteristic peaks on the pyrogram shown in Fig. 1

Peak number	Compound	Structure of the original fatty acid, (*i*:*j*)[a]	ECN[c]
1	Methyl tetradecanoate (myristate)	14:0	13.95
2	Methyl hexadecanoate (palmitate)	16:0	15.95
3	Methyl *cis*-7-hexadecenoate	16:1(*cis*-7)	15.85
4	Methyl *cis*-9-hexadecenoate (palmitoleate)	16:1(*cis*-9)	15.85
5	Methyl octadecanoate (stearate)	18:0	17.95
6	Methyl *cis*-9-octadecenoate (oleate)	18:1(*cis*-9)	17.85
7	Methyl *cis*-11-octadecenoate	18:1(*cis*-11)	17.85
8	Methyl octadecadienoate[b]	18:2	17.75
9	Methyl octadecadienoate[b]	18:2	17.75
10	Methyl octadecadienoate[b]	18:2	17.75

[a] The number 18:1, for example, indicates the carbon number (18) and one double bond.
[b] Position of double bond was not specified.
[c] Empirically estimated values based on the structures.

reagent. Table 1 summarizes the assignment of these characteristic peaks together with their abbreviations and effective carbon numbers (ECNs), which correspond to the relative molar sensitivity of the FID [18]. As shown in Table 1, these peaks were assigned to the methyl esters of saturated and unsaturated C_{15} to C_{18} fatty acids resulting from the selective hydrolysis of ester bonding in the lipids followed by simultaneous methylation during the pyrolysis in the presence of TMAH. Furthermore, Py–GC measurements of other *Daphnia* and algae samples also yielded similar pyrograms, on which the series of fatty acid methyl esters from C_{15} to C_{18} were clearly observed. These results indicate that Py–GC in the presence of TMAH enables highly sensitive detection of fatty acid components up to C_{18} contained either in a single zooplankter or micro amounts of algae sample as their methyl esters. However, the fatty acid components longer than C_{20}, which were known to be contained in zoo- and phytoplankton [1,2] mainly as highly unsaturated fatty acids, could not be detected on the pyrogram due to their isomerization and/or degradation caused by TMAH. Furthermore, as described in our preceding report [15], the relative standard deviation of the distribution for 18:2 fatty acids obtained by this method showed relatively higher values (14.2%) owing to their much more complex isomerization. Therefore, in this work, the empirically observed fatty acid compositions from 14:0 to 18:1 by the proposed technique were used as the database for the principal component analysis in order to differentiate each plankton sample.

According to the method proposed in our previous paper [15], the apparent distributions of the fatty acid components were also calculated on the basis of peak intensities of fatty acid methyl esters on the resulting pyrograms using the following equation:

$$\text{fatty acid composition (mol\%)} = \frac{P_{i:j}/\text{ECN}_{i:j}}{\sum(P_{i:j}/\text{ECN}_{i:j})} \times 100$$

where $P_{i:j}$ and $\text{ECN}_{i:j}$ are the observed peak intensity and the ECN for the methyl ester of $C_{i:j}$ fatty acid containing i carbon(s) and j double bond(s), respectively. Table 2 shows the observed chemical compositions from 14:0 to 18:1 obtained for a single individual measurement of fifteen *Daphnia* samples and three repeated measurements for each algae sample. These data suggest that the observed fatty acid compositions of every *Daphnia* sample have close correlations to those of the ingested algae sample. For example, for *Daphnia* samples cultured with *Chlorella* and *Scenedesmus*, the relative abundances of 16:0 and 18:1(*cis*-9) are significantly higher than those of other fatty acid components reflecting those for the ingested algae phytoplankton samples. Similarly, the significantly high abundance of 16:0 in *Chlamydomonas* is preserved in corresponding *Daphnia* samples. Nevertheless, the positive and/or negative discrepancies between the ob-

Table 2
Observed chemical composition of *Daphnia* individuals and ingested algae samples

	Fatty acids							
	14:0	16:0	16:1(*cis*-7)	16:1(*cis*-9)	18:0	18:1(*cis*-9)	18:1(*cis*-11)	
	Compositions (mol%)							Total
Daphnia	5.53	37.1	11.2	13.7	4.59	16.1	11.7	100
cultured by	4.98	33.1	13.0	13.2	5.07	19.2	11.5	100
Chlamydomonas	4.86	32.0	12.4	13.7	6.00	19.6	11.4	100
	5.15	32.9	11.9	15.8	5.76	17.0	11.4	100
	5.33	37.5	10.6	16.6	3.85	14.5	11.5	100
Mean values	5.17	34.5	11.8	14.6	5.05	17.3	11.5	100
Chlamydomonas	3.45	63.1	5.19	6.77	2.71	1.87	16.8	100
	3.43	62.7	5.48	6.77	2.77	2.09	16.8	100
	2.89	66.6	5.94	6.98	2.10	1.91	13.6	100
Mean values	3.26	64.1	5.54	6.84	2.53	1.96	15.7	100
Daphnia	2.91	26.3	17.6	8.59	8.59	27.0	8.98	100
cultured by	3.66	28.3	19.7	8.31	8.41	23.5	8.03	100
Chlorella	3.24	26.8	18.0	8.88	6.60	27.3	9.27	100
	3.44	27.8	17.3	8.55	6.14	27.1	9.58	100
	3.14	27.0	17.9	10.1	6.68	25.6	9.50	100
Mean values	3.28	27.2	18.1	8.89	7.28	26.1	9.07	100
Chlorella	1.98	34.1	10.8	2.27	2.25	39.6	9.03	100
	1.73	34.6	10.9	2.34	2.02	39.4	8.99	100
	1.62	33.5	10.3	2.11	1.44	40.9	10.1	100
Mean values	1.75	34.1	10.7	2.24	1.90	40.0	9.37	100
Daphnia	3.01	27.5	17.3	4.34	4.99	38.0	4.75	100
cultured by	2.75	26.8	16.8	4.03	4.88	40.5	4.27	100
Scenedesmus	3.01	26.5	17.5	4.01	4.84	39.6	4.55	100
	2.96	27.0	17.5	4.03	4.54	39.9	4.07	100
	2.97	28.0	17.6	4.27	4.51	38.4	4.15	100
Mean values	2.94	27.2	17.3	4.14	4.75	39.3	4.36	100
Scenedesmus	0.84	45.0	7.41	–	1.21	44.3	1.24	100
	0.67	36.9	5.54	–	1.34	54.3	1.31	100
	0.81	44.0	6.65	–	1.22	45.9	1.36	100
Mean values	0.77	42.0	6.53	–	1.26	48.2	1.30	100

served fatty acid compositions for a given *Daphnia* and the corresponding algae used as the food, imply a fairly strong contribution of isomerization, degradation and/or elongation of a part of fatty acid components during digestion in *Daphnia* individuals after intake by the zooplankter.

Therefore, in order to take all the observed compositional information into consideration, PCA was applied to the observed compositional values for the evaluation of the relationships between a given *Daphnia* group and the corresponding algae samples used as food. Table 3 shows the list of the first and second principal component scores (PC1 and PC2, respectively) and their two-dimensional visualization is shown in Fig. 2. These statistical data were calculated from those resulting from a single measurement for *Daphnia* individuals and a single measurement for algae samples. PC1 and PC2 describes 50.0 and 39.9% of the total variance, respectively. In Fig. 2, the plots for algae samples are discretely separated into three groups according to the species, while those for *Daphnia* samples are

Table 3
First and second principal component scores of zooplankter individuals and ingested algae samples

(a) *Daphnia* individual samples

	Daphnia cultured by *Chlamydomonas*					*Daphnia* cultured by *Chlorella*					*Daphnia* cultured by *Scenedesmus*				
PC1	62.4	60.9	61.4	63.4	63.1	54.7	56.2	54.1	54.1	55.0	45.7	44.2	45.0	44.4	44.8
PC2	48.5	51.0	51.9	51.2	47.4	59.0	59.8	57.2	56.1	57.1	57.0	57.0	57.4	57.1	56.8

(b) Algae samples ingested by *Daphnia* samples

	Chlamydomonas			*Chlorella*			*Scenedesmus*		
PC1	58.2	58.2	55.3	41.7	41.3	40.8	32.7	30.4	32.3
PC2	33.3	33.6	33.1	47.5	47.2	46.4	44.3	46.1	44.2

also clearly separated into the other three groups reflecting the algae species used as food. Here, it is worthy to note that the interindividual differences of *Daphnia* samples are much smaller than their differences due to the kinds of food as shown in this figure. Furthermore, the score plots for *Daphnia* individuals are shifted in parallel by almost the same increment compared to those for ingested algae samples. This fact suggests that lipid biosynthesis such as isomerization and/or elongation of fatty acid components occurs in a similar ratio for these *Daphnia* individuals independently of the species of ingested algae phytoplankton. These results indicate that the fatty acid compositions of *Daphnia* individuals used in this work are significantly reflected in those of their food although isomerization and/or elongation of fatty acid components occur during digestion to some extent.

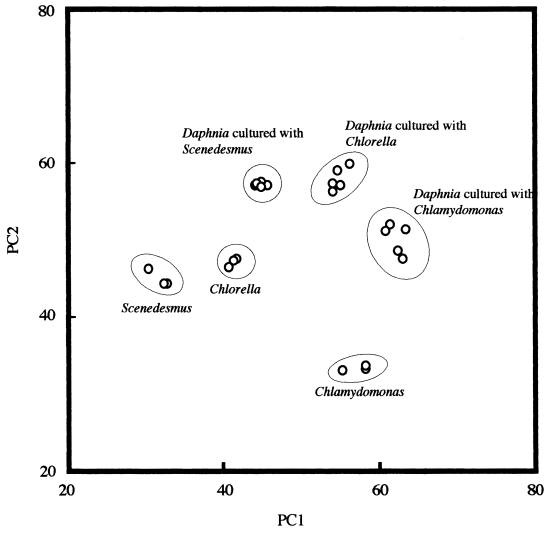

Fig. 2. First and second principal component scores for each *Daphnia* sample and ingested algae sample.

In this work, the relationships between fatty acid compositions in the lipids of a given zooplankter and those for the ingested algae were evaluated by use of PCA based on the observed fatty acid composition obtained by the Py–GC technique combined with on-line methylation. As for the isomerization and/or degradation of polyunsaturated fatty acids, it was recently proven that the use of trimethylsulfonium hydroxide (TMSH) as the chemical reagent instead of TMAH allowed detection of polyunsaturated fatty acid residues up to 22:6 in various oil samples by our work [19]. The application of Py–GC in the presence of TMSH to lipid analysis in zooplankter samples is currently in progress.

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