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Discriminative Analysis of Zooplankton Individuals by Pyrolysis–Gas Chromatography Combined With On-line Methylation

Yasuyuki Ishida, Shinich Isomura, Shin Tsuge, Hajime Ohtani, Tatsuki Sekino, Masami Nakanishi, and Takashi Kimoto

Department of Applied Chemistry, School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan
Division of Materials Science, Centre for Integrated Research in Science and Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan
Centre for Ecological Research, Kyoto University, Shimosakamoto, Otsu 520-01, Japan
Research Institute of Oceanology and Chemistry, Funabashi-cho, Tenouji-ku, Osaka 543, Japan

Discriminative analysis between each zooplankton individual was achieved on the basis of observed total lipid contents and their fatty acid compositions obtained by pyrolysis–gas chromatography combined with on-line methylation using tetramethylammonium hydroxide (TMAH). Pyrolysis in the presence of TMAH allowed the highly sensitive detection of fatty acids in one zooplankton individual as their methyl esters on the resulting pyrogram. The distributions of each saturated and monounsaturated fatty acid, estimated with relative standard deviations of less than 4.5%, were in good agreement with those determined by conventional methylation followed by GC using powdered plankton samples prepared by cryo-milling homogenization. The peak intensities of fatty acid components on the pyrograms were successfully used to discriminate each plankton individual, which differ in the food concentrations, without applying any complicated pre-treatment. The results obtained suggest that plankton individuals cultured in higher food concentrations contain lipid contents between 7.3 and 8.9% m/m whereas those cultured in lower food concentrations lie between 3.2 and 5.9% m/m.

Keywords: Pyrolysis–gas chromatography; on-line methylation; zooplankton individuals; lipid analysis; discriminative analysis

Introduction

The amounts of total lipid and its fatty acid composition in an individual zooplankton vary, depending not only on the species of the plankton but also on various conditions such as the degree of its growth and reproduction, seasonal changes, geographic origin and sampling depth. Therefore, it is important to determine the amounts of lipid and the related fatty acid composition in an individual zooplankton in order to elucidate the correlations between those parameters and the environmental conditions. Their determination in every zooplankton individual is of interest in order to obtain detailed information about its life history, the structure of the population and population dynamics. Conventional methods, however, are not applicable mainly because of the very minute sample size: a plankton individual weighs only a few micrograms.

In general, the lipid content and the fatty acid composition of plankton samples are determined by GC or LC with preliminary treatment including extraction and transmethylation. Farkas determined the fatty acid composition of the total lipids and of some lipid classes of two planktonic crustacean species by extraction and methylation followed by GC. Lee et al. evaluated the effects of changes of the food type and food concentration on the amount of lipids and the composition of wax esters in phytoplankton and copepods by GC after solvent extraction of the samples (approximately 6 mg) followed by saponification and derivatization. However, this technique cannot be applied to the lipid characterization of a zooplankton individual because of the requirement for fairly large amounts of zooplankton for the extraction procedure.

Visual measurements of the lipid in cladocerans have been widely used to evaluate the lipid content of a zooplankton individual based on the number and size of lipid droplets in each body. However, discriminative analysis among zooplankton individuals, which differ slightly in the environmental conditions, cannot be achieved by use of visual measurements, which provide a relatively rough estimation of the lipid amounts.

On the other hand, analytical pyrolysis techniques such as pyrolysis–mass spectrometry (Py–MS), Py–GC and Py–GC–MS have been successfully applied in various fields, including microbiology, organic geochemistry and environmental chemistry. Previously, the chemical characterization of organic matter in the marine environment, such as aquatic plants, zooplankton, microorganisms and suspended matter in the ocean, was carried out by Py–MS combined with multivariate analysis. Although this technique provided a fairly sensitive method to evaluate the organic matter by use of minute amounts of samples of the order of 10 μg, there has been no report on discriminative analysis among plankton individuals. Further, conventional Py–GC or Py–GC–MS, which might offer an alternative technique, does not necessarily provide effective information about the lipid component of zooplankton because of the very weak intensity of the characteristic peaks of the fatty acid components, mainly owing to their high polarity.

Recently, it has been shown that reactive pyrolysis of condensation polymers in the presence of an organic alkali, such as tetramethylammonium hydroxide (TMAH), results in a simplified pyrogram which consists of characteristic peaks of methyl derivatives reflecting the constituents of the original polymers. We previously applied this technique to the compositional analysis of aromatic polyesters and the determination of rosin sizing agents in paper samples. Further,
the mechanism of the pyrolysis of the aromatic polyester in the presence of TMAH was studied in detail by the use of Py–GC and Py–GC–MS.13 This technique, termed 'thermally assisted chemolysis (TAC),14 has also been successfully applied to the fine structure analysis of trace amounts of biological and geological samples containing ester linkages in their polymer chain.14–17

In this work, Py–GC in the presence of TMAH was applied to the determination of the lipid contents and their fatty acid compositions of every zooplankton individual. Further, discriminative analysis among zooplankton individuals cultured in different food concentrations was carried out on the basis of the peak intensities of the fatty acid methyl esters on the pyrograms.

Experimental

Materials

Daphnia galeata samples cultured in the laboratory were used for the lipid analysis. Cultured samples were classified into two groups according to the food concentration. Four Daphnia individuals (H group) were cultured in 10^6 Chlorella cells ml\(^{-1}\) (the high food cultures), yielding individual dry masses from 6 to 25 µg each. The other five Daphnia individuals (L group), ranging between 10 and 15 µg, were kept without any food for 5 days after being cultured in 10^6 Chlorella cells ml\(^{-1}\). Further, a finely powdered plankton sample consisting of three species of natural zooplankton (Daphnia, Bosmia and Eodiaptomus) was prepared by cryo-milling homogenization at liquid nitrogen temperature as a standard to establish the precision of this method. These plankton samples were collected in Lake Biwa in 1984.

Analytical-reagent grade tripalmitin, supplied by Wako (Osaka, Japan), was used as a standard triglyceride to determine the fatty acid content in plankton.

An aqueous solution (25% m/m) of tetramethylammonium hydroxide (TMAH), supplied by Tama Chemicals (Tokyo, Japan), was used as a reagent for pyrolysis combined with on-line methylation.

Procedure for Extraction and Transmethylation

The results obtained using Py–GC in the presence of TMAH were compared with those for the conventional GC method after extraction and transmethylation, using a powdered plankton sample. Lipid extraction of the sample was performed according to the Bligh–Dyer method.18 The total lipid content was obtained from the mass of the extracted lipid. Fatty acid methyl esters, prepared by transmethylation of extracted lipids with methanol containing 10% HCl in an acid digestion bomb at 100 °C for 1 h, were extracted with hexane and then subjected to GC measurement.

Conditions for Py–GC

The procedure for Py–GC combined with on-line methylation is basically the same as described previously.11–13 Fig. 1 shows a schematic flow diagram of the measuring system for Py–GC together with the sampling procedure. A vertical microfurnace pyrolyser, B, carrier gas inlet (He); C, make-up gas (N\(_2\)); D, splitter; E, glass insert tube; F, fused-silica capillary column (FSCC); G, flame ionization detector (FID); H, platinum sample cup; I, column oven; J, tetramethylammonium hydroxide (25% m/m aqueous solution, 2 µl); K, one zooplankton individual.

Injection of fatty acid methyl esters after transmethylation, about 2 µl of hexane solution was directly introduced into the heated centre of the pyrolyser maintained at 240 °C. A fused-silica capillary column (Hewlett-Packard Ultra 1, 25 m × 0.2 mm id) coated with polydimethylsiloxane (0.33 µm film thickness), immobilized by chemical cross-linking, was used for GC. The 50 ml min\(^{-1}\) carrier gas flow rate at the pyrolyser was decreased to 1.0 ml min\(^{-1}\) at the capillary column by means of a splitter. The column temperature was initially set at 50 °C and then programmed to 280 °C at 5 °C min\(^{-1}\). Identification of the peaks on the pyrograms was carried out mainly using a GC–MS system, Automass 150, (JEOL, Tokyo, Japan) with an electron impact ionization (70 eV) source to which the pyrolyser was directly attached. The resulting data were processed by use of principal component analysis as described by Mitsui et al. in order to differentiate nine plankton individuals by considering all the fatty acid components observed on the pyrograms.

Results and Discussion

Fig. 2 shows typical pyrograms obtained from cultured Daphnia individuals weighing about (a) 11 µg and (b) 21 µg at 400 °C,
obtained from the mass of extracted lipids. Not detected.

Typical patterns for polyunsaturated fatty acid methyl ester. Py-GC 15.75 46.78 10.45 8.45 18.99

TMAH. Similarly, characteristic peaks 2-7 were assigned to the pyrograms obtained without adding the reagent, a series of sharp peaks (1-7) are evident in Fig. 2(b) after the elution of trimethylamine and methanol formed from TMAH solution. Additionally, the elution of methyl ethers of glycerol at 5 min indicates the presence of triglyceride in individual plankton. The mass spectrum of peak 1 includes a molecular ion peak at m/z 242 and fragment ion peaks such as those at m/z 213, 199, 143, 129 and 87 together with the base peak at m/z 74. Therefore, peak 1 can be assigned to methyl tetradecanoate (14:0), which was formed from the lipid components in the plankton individual through transmethylation in the presence of TMAH. Similarly, characteristic peaks 2-7 were assigned to the methyl esters of saturated and unsaturated C15-C18 fatty acids. As shown above, Py-GC in the presence of TMAH allows the highly sensitive detection of fatty acid components contained in a zooplankton individual as their methyl esters.

The accuracy and precision of the method were determined using a powdered plankton sample prepared by cryo-milling homogenization. The values of total lipid observed by Py-GC in the presence of TMAH were obtained using the peak intensities of methyl esters of saturated and unsaturated C15-C18 fatty acids. The values in bold type are the number of corresponding segments and/or structure in a molecule of the methyl ester. Then, provided that all the fatty acids in a given zooplankton sample exist in triglyceride form and are converted into the methyl esters, the total lipid content in grams for each plankton sample is calculated as follows:

\[
\text{total lipids} = \sum_{i=14}^{18} \sum_{j=0}^{2} m_{ij} \times \frac{1}{M_{ij}} \times \frac{1}{3} \times (3 \times M_{ij} - 4)
\]

where the factor 1/3 is the reciprocal of the number of methyl esters of fatty acids formed from each molecule of the related triglyceride stoichiometrically through the reactive pyrolysis and the value (3 \times M_{ij} - 4) is the molecular mass of the associated triglyceride.

Table 1 shows the observed total lipid contents and the distributions of saturated and monounsaturated fatty acid components detected both by Py-GC and by GC after solvent extraction followed by transmethylation for the common powdered plankton sample. Values in square brackets represent the mean distributions for all the fatty acid components detected by both methods for five measurements. It should be noted that the observed distributions of fatty acids and the total lipid contents obtained by Py-GC and conventional GC are fairly different from each other even after consideration of the high relative standard deviations (e.g., 5.9%) for the Py-GC measurements. In particular, polyunsaturated fatty acid components are strongly depressed in Py-GC because the thermal degradation of such fatty acids could occur during the pyrolysis in the presence of TMAH. However, fairly good agreement is observed between the relative distributions among commonly observed saturated and monounsaturated fatty acid components by both methods (14:0 to 18:1). Further, the fact that the relative standard deviations for the distributions of saturated and monounsaturated fatty acids obtained by Py-GC are less than

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<th>Fatty acid</th>
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<td>Method</td>
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<tr>
<td>Py-GC</td>
<td>15.7†</td>
</tr>
<tr>
<td>Conventional GC</td>
<td>13.0†</td>
</tr>
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* 16:1 isomers which differ in the position of double bond. † The molecular mass was not confirmed by their mass spectra, all of which indicated typical patterns for polyunsaturated fatty acid methyl ester. ‡ Total lipid (% m/m) was calculated against dry mass of powdered plankton sample. § Chemical compositions among 14:0, 16:0, 16:1b, 18:0 and 18:1 fatty acids, which were commonly observed in both methods. ¶ Values in parentheses are relative standard deviations for five measurements. † Values in square brackets are observed levels of all the fatty acid components detected by each method; nd = not detected. ** Observed total lipid content calculated from peak intensities of fatty acid components on the pyrogram using eqn. (4). *** Total lipid content obtained from the mass of extracted lipids.
4.5% indicates that the precision of this method is good enough to use for the discrimination of each zooplankton individual which differ in environmental conditions. Py-GC in the presence of TMAH at 400 °C was then applied to differentiate zooplankton individuals cultured in different food concentrations. Table 2 shows the distributions of each fatty acid component and the observed total lipid contents for nine plankton individuals. The distributions of each fatty acid component are similar among these nine individuals regardless of the difference in the food concentration. In addition, these plankton individuals contain 3–4% of C15:0 fatty acid, which was reported to be present in only negligibly small amounts in previous papers. This component might have been introduced into the culture medium by some bacteria containing C15:0 fatty acid. It is worth noting that the observed total lipid contents (% m/m) for the H series are about double those for the L series, although the compositional distribution of the fatty acids are almost the same between H and L series.

Principal component analysis was then carried out in order to differentiate the nine plankton individuals considering all the fatty acid components observed on the pyrograms. The observed peak intensities for methyl esters of fatty acids normalized by sample mass were used as the database after being converted into deviation values in order to equalize the food concentrations.

In conclusion, Py-GC combined with on-line methylation proved to be a rapid, convenient and highly sensitive method to evaluate the total lipid content and corresponding fatty acid composition in every plankton individual of dry mass 6–25 μg within 1 h and without applying any complicated pre-treatment. In order to achieve the determination of all the fatty acid components contained in a zooplankton individual, further optimization of the pyrolysis conditions is needed. Detailed comparative studies of lipid distributions in various plankton obtained from different environments are in progress.

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### Table 2

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<th>Fatty acid</th>
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<td>14:0</td>
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<td>15.0</td>
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<td>15:0</td>
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<tr>
<td>18:2</td>
<td>16.8</td>
<td>18:2</td>
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- Relative % m/m among the observed fatty acid components.
- Total lipid (μg) was calculated assuming that all the fatty acids exists in triglyceride form in the zooplankton sample.
- Values in parentheses are dry masses (μg) measured by microbalance.

### References