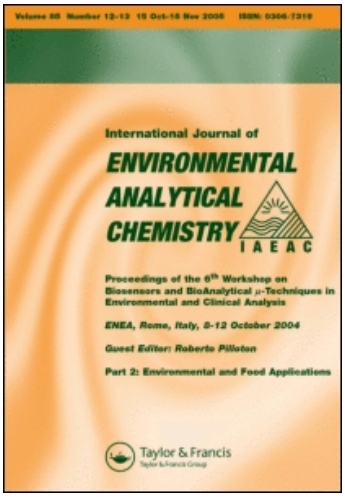

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A METHOD FOR MICRO-DETERMINATION OF TOTAL MICROCYSTIN CONTENT IN WATERBLOOMS OF CYANOBACTERIA (BLUE-GREEN ALGAE)

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Microcystins as cyanobacterial (blue-green algal) toxins are found from toxic waterblooms in freshwater lakes and reservoirs for drinking water all over the world.

In this paper, we demonstrated a micro-determination method for total microcystin content in waterblooms of toxic cyanobacteria. This method based on a quantitative analysis of 2-methyl-3-methoxy-4-phenylbutyric acid(MMPB) as an oxidation product of microcystins by gas chromatography(GC) with a flame ionization detector or high performance liquid chromatography(HPLC) with a fluorescence monitor. In the case of GC analysis, methyl ester of MMPB in the pmol range of concentration was determined. On the other hand, in the HPLC analysis, MMPB in the fmol range of concentration was determined by labeling with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate as a fluorescent reagent.

These results pointed out that this micro-analysis is able to be applied to the monitoring of microcystins in drinking water, reservoirs and freshwater lakes.

KEY WORDS: Microcystins, 2-methyl-3-methoxy-4-phenylbutyric acid, GC, HPLC, cyanobacteria(blue-green algae).

INTRODUCTION

Waterblooms of toxic cyanobacteria (blue-green algae) have been detected in freshwater lakes and reservoirs for drinking all over the world[1,2,3]. These blooms were reported as causing death to domestic animals after drinking affected water[4,5], and as a potential hazard to human health[6,7]. Toxic cyanobactria produce hepatotoxins named microcystins. The general structure for the toxins has been described as cyclo(D-Ala-L-X-*erythro*-b-methyl-D-*iso*-Asp-L-Y-Adda-*iso*-Glu-N-methyldehydro-Ala) where Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and X and Y refer to variable amino acids[8,9,10].

Recently, Fujiki and his coworkers[11] found that microcystins have activities of inhibition of protein phosphatases and tumor promotion, in the nanomolar range of concentration. Their findings suggest that micro-analysis of microcystins is urgently required for drinking water. The analysis of microcystins has been performed by high performance liquid

chromatography(HPLC) with a UV detector using reverse-phase and/or ion-exchange columns[12, 13, 14]. However, the sensitivity of the detection method was not satisfactory, since the molecular absorption coefficient of the toxins is rather low (λ max 239 nm (log ε = 4.49)[15].

Chu *et al*[16,17] have developed a method for the analysis of microcystins using enzyme-linked immunosorbent assay (ELISA) with microcystin LR antibody. However, the antibody has different specificities against each microcystin variants, so the total microcystin content in the mixture of microcystins couldn't be determined by the ELISA method.

In this paper, we have designed a micro-determination method for total microcystin content in waterblooms. This method is based on a quantitative analysis of 2-methyl-3-methoxy-4-phenylbutyric acid as an oxidation product of Adda in microcystin molecules by gas chromatography (GC) with a flame-ionization detector (FID) or HPLC with a spectrofluorometer.

MATERIALS AND METHODS

Microcystin RR and LR were purified from an axenic clonal strain of *Microcystis viridis*(NIES 102) isolated from Lake Kasumigaura by a method designed in a previous paper[14]. Toxic waterblooms were collected from Lake Kasumigaura in the summer of 1990, and were lyophilized and stored at -80 °C. 4-Phenylbutyric acid, other reagents and solvents were purchased from Wako Pure Chemicals Ltd.

Preparation of microcystins fraction

Microcystins were extracted with 5% acetic acid[13] from lyophilized waterblooms, and were fractionated using C18 Sep-Pak Cartridge[12].

Preparation of 2-methyl-3-methoxy-4-phenylbutyric acid(MMPB)

Microcystins fraction(about 0.2-1.0 mg) was dissolved with 0.2 ml of 90% Acetic acid, and was reacted with an oxidant solution containing 0.02 M sodium metaperiodate and 0.024 M potassium permanganate[20]. The reaction mixture was shaken for 4 hr at room temperature. When the color of the $KMnO_4$ ion disappeared during this period, the oxidant solution was added until the color remained. The mixture was then decolorized by addition of 0.04 ml of 20% sodium bisulfite solution. After addition of 0.01 ml of 10M H_2SO_4, MMPB was extracted with 5 ml of ethyl acetate.

Methyl ester of MMPB

MMPB was converted to methyl ester using 14% trifuoroborate methanol solution[19].

Fluorescent label of MMPB

MMPB was reacted with 2-(2,3-maphthalimino)ethyl trifluoromethanesulfonate(NE-OTf) in acetonitrile containing 18-crown-6 and anhydrous potassium fluoride[18].

Internal standard

4-Phenylbutylric acid(0.01 - 0.1 mmol) as an internal standard was added into the microcystins fraction before the oxidation.

GC analysis

The methyl esters were analyzed in HP-5890(Hewlett-Packard, U.S.A.) using a HP-1 capillary column(0.32 mm × 25 m) (Hewlett-Packard, U.S.A.). The chromatograph was equipped with a flame ionization detector (FID), and was temperature-programmed. The temperature was hold at 80 °C for 1.5 min, then heated to 260 °C at the rate of 8 °C min^{-1}. Peaks were automatically integrated using a computer integrator.

HPLC analysis

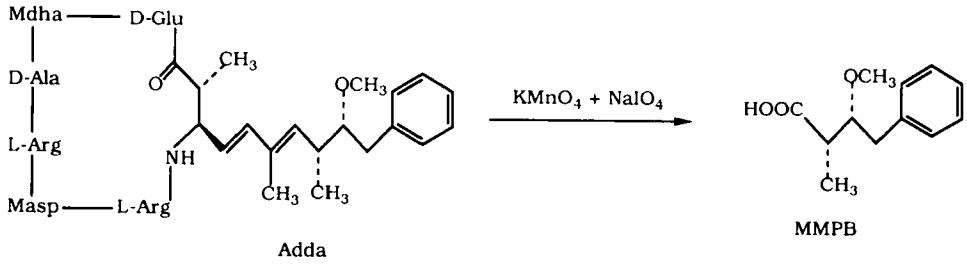
Fluorescent labeled compounds were analyzed in a Shimadzu LC-6A (Shimadzu Corp. Kyoto, Japan) with a fluorescence monitor(Shimadzu RF 535) and a Nova-Pak C18 column(3.9 mm × 15 cm) using a mobil phase of methanol/water(7/3) at the flow-rate of 1.0 ml min^{-1}. Peaks were detected at Em 394 nm (Ex 259 nm), and were automatically integrated using a computer integrator(Shimadzu C-R4A).

MS analyses

Electron impact(EI) and chemical ionization(CI)-MS spectra were recorded on a JEOL JMS DX-300 mass spectrometer (JEOL, Tokyo, Japan). MS data were obtained under the following conditions: ionization current, 300 μA; ionization energy, 70 eV; accelerating voltage, 3 kV; scan range, 30-500 m/z. In the case of CI-MS, iso-buthane was used as a reagent gas.

RESULTS AND DISCUSSION

Microcystins contain one molecule of Adda in their molecules. By the oxidation of microcystins using potassium permanganate and sodium metaperiodate, Adda residue should be oxidized to the corresponding carboxylic acid derivative(MMPB). To confirm the formation of MMPB by oxidation of microcystin samples, the oxidized products of



Scheme 1 Formation of MMPB(2-methyl-3-methoxy-4-phenylbutyric acid) from Adda(3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) residues present in microcystins.

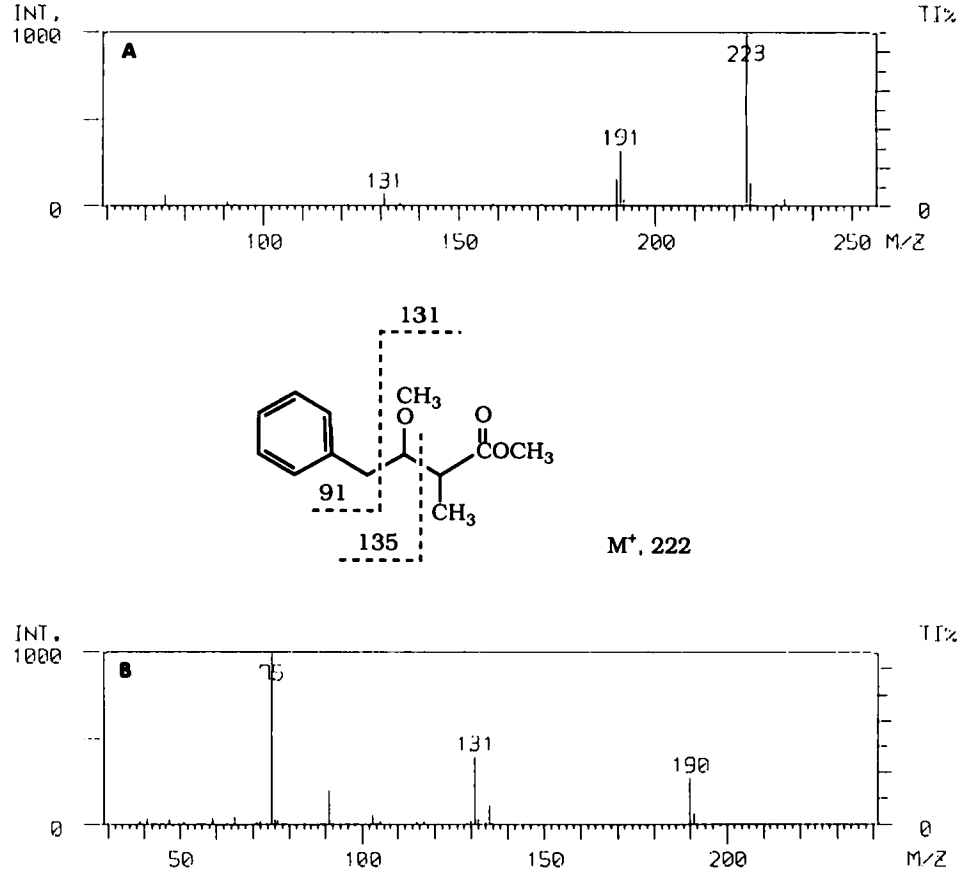


Figure 1 CI-MS and EI-MS spectra of MMPB(2-methyl-3-methoxy-4-phenylbutyric acid). The CI-MS spectrum(A) show the $(M + 1)^+$ ion at m/z 223. The EI-MS spectrum(B) does not present the molecular ion(M^+, 222), but exhibits an M^+ - 32 ion at m/z 190(loss of CH_3OH). Assignments of fragement ions are shown in the figure.

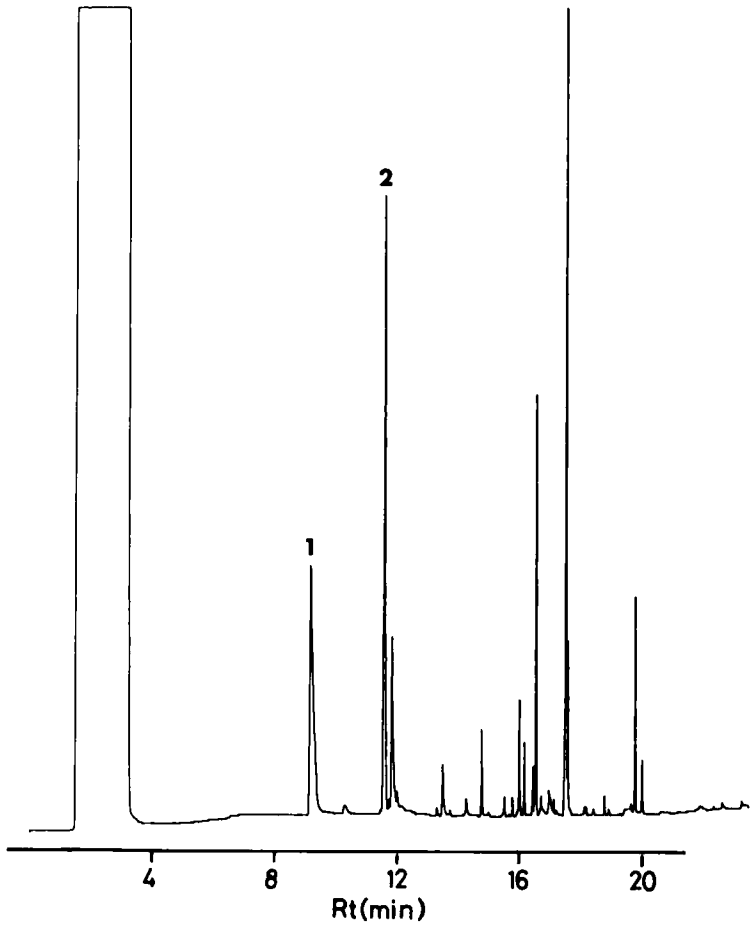


Figure 2 Gas chromatogram of MMPB prepared from toxic waterblooms (sample No. 1). The operation conditions of GC are described in the Materials and Methods section. Peak 1 and 2 are the internal standard 4-phenylbutyric acid methyl ester (25 pmol) and the methyl ester of MMPB, respectively.

microcystin RR were converted to methyl ester, and analyzed by GC-MS. When the methyl ester of the oxidized product was analyzed by GC with a capillary column, a large peak was detected at 11.6 min. In the CI-MS spectrum of the peak, the $(M + 1)^+$ ion was observed as the largest ion peak at m/z 223. This value agreed with the methyl ester of MMPB($C_{13}H_{18}O_3$, M_r,222). As example an EI-MS spectrum of the methyl ester is shown in Figure 1. The molecular ion was not present, but the $(M - 32(MeOH))^+$ ion could be detected at m/z 190. Also recorded were the ions at m/z 135, 131, 91 and 75(base peak). Probably, the base peak ion could be assigned a structure such as CH_3-C(=O^+-H)-OCH. These results of MS spectra prove that the methyl ester of the oxidation product of microcystin RR was always MMPB.

When a small amount of microcystin RR(0.1 mmol) was oxidized at room temperature for 4 hr, the yield of MMPB was 84% of the theoretical amount which was determined by the GC method described in the Materials and Methods section. Whereas decomposed-

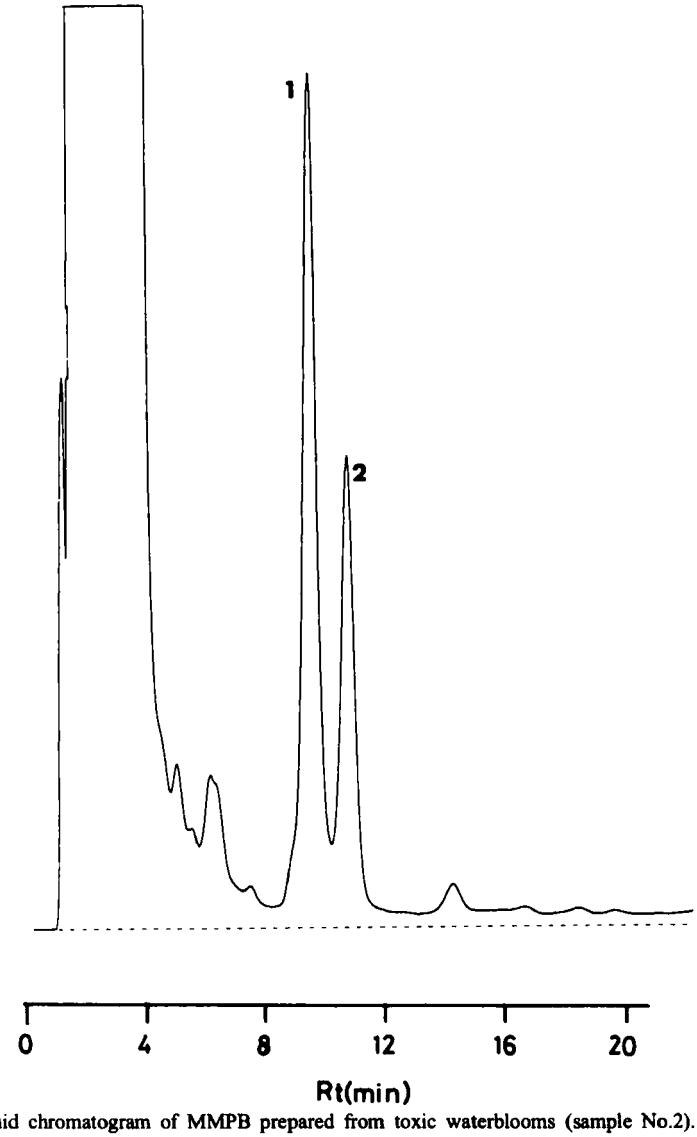


Figure 3 Liquid chromatogram of MMPB prepared from toxic waterblooms (sample No.2). The operation conditions of HPLC were described in the Materials and Methods section. Peak 1 and 2 are MMPB and the internal standard 4-phenylbutyric acid (250 fmol), respectively.

microcystin RR was 98%, determined by HPLC14. To examine if this fact was due to poor sample extraction recovery of MMPB, an authentic sample of MMPB*(0.1 mmol) was

**Synthesis of MMPB* Methyl propionate and lithium diisopropylamide were mixed in tetrahydrofuran at -70 °C. Then, 2-phenylacetoaldehyde was added into the mixture. 3-hydroxy-2-methyl-4-phyenylbutric acid methyl ester as a precursor of MMPB formed in the reaction mixture, was separated by silica gel column. The precursor was converted to MMPB by methylation 21 and saponification. (Sodium salt of MMPB, $C_{12}H_{15}O_3Na$, Mr 230, λmax (H_2O) 253nm (log ε = 1080))

Table 1 Comparison of microcystin content analysis of toxic waterblooms by GC and HPLC. Toxic waterblooms(sample No.1 and 2) were collected from Lake Kasumigaura. The analytical methods were described in the Materials and Methods section. Results were expressed as pmol mg^{-1} ± S.D.(n = 3) lyophilized waterblooms.

	GC	*HPLC*
Sample No.1	278.34 ± 7.02[a)]	281.63 ± 9.10[a)]
Sample No.2	4.72 ± 0.24[a)]	4.79 ± 0.17[a)]

[a)]Differences between the analyses of GC and HPLC were not statistically significant by Student's *t* test.

dissolved into 90% acetic acid solution, then oxidized and extracted with ethyl acetate. The recovery of MMPB was 86 ± 4%. In the case of 4-phenylbutyric acid always used as an internal standard, the recovery(87 ± 5%) was also the same as that of MMPB.

In order to determine the microcystin content in toxic waterblooms, natural samples, the microcystins fraction was separated from the lyophilized waterblooms by the method described in the Materials and Methods section. The fraction containing the internal standard 4-phenylbutyric acid(0.01- 0.1 mmol) was oxidized. The oxidation products were extracted after the addition of 10M H_2SO_4. Then, carboxyl group-containing compounds in the extract were converted to methyl esters or labeled with the fluorescent labeling reagent, and submitted to chromatographic analysis.

A typical GC chromatogram of the methyl esters prepared from the waterbloom sample (No.1) is shown in Figure 2. Peak 1 and 2 were assigned as methyl 4-phenylbutyric acid (25 pmol) and methyl ester of MMPB, respectively. Moreover, by GC-FID analysis, MMPB in the pmol range of concentration could also be detected. In addition, HPLC analyses of fluorescent labeled MMPB, waterblooms sample (No.2) and 4-phenylbutyric acid (250 fmol) were determined by HPLC using a reverse-phase column and a fluorescence detection (Figure 3.) By this method, MMPB in the fmol range of concentration was determined. The results are summarized in Table 1. When the values of microcystin contents obtained from the HPLC analysis compared with those of the GC analysis, statistically no significance was obtained between HPLC and GC analysis by Student's *t* test.

This micro-analysis can also detects other Adda containing toxins such as nodularin which contains in cells of *Nodularia spumigena*[10,22]. These results proved that these techniques may be applied to the monitoring Adda-containing toxins in drinking water, reservoirs and freshwater lakes.

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