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Short communication

Analytical aspects of cyanobacterial volatile organic compounds for investigation of their production behavior

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ABSTRACT

In order to fully understand the role of volatile organic compounds (VOCs) under natural conditions, an adaptable analytical method was developed as the first step. β-Ionone, β-cyclocitral, 2-methyl-1-butanol and 3-methyl-1-butanol were simultaneously analyzed in addition to geosmin and 2-MIB using GC/MS with SPME. The slight modification of a known method allowed the simultaneous detection and quantification of these VOCs. The SIM of the 3-methyl-1-butanol was always accompanied by a shoulder peak, suggesting the presence of two compounds. In order to separate both compounds, the GC/MS conditions were optimized, and the additional peak was identified as 2-methyl-1-butanol by direct comparison of the authentic compound, indicating that the *Microcystis* strain always produces a mixture of 2-methyl-1-butanol and 3-methyl-1-butanol. Furthermore, it was found that 2-methyl-1-butanol and 3-methyl-1-butanol were predominant in the dissolved fractions. β-Cyclocitral was easily oxidized to provide the oxidation product, 2,6,6-trimethylcyclohexene-1-carboxylic acid, which causes the blue color formation of cyanobacteria as a consequence of acid stress. The intact acid could be satisfactorily analyzed using the usual GC/MS without derivatization.

1. Introduction

Blooms and scums of cyanobacteria (blue-green algae) commonly occur in lakes, reservoirs, slow-flowing rivers and brackish water environments, resulting in the water having a musty odor [1] and the production of potent toxins [2]. Many taste and odor outbreaks have been associated with volatile organic compounds (VOCs), such as geosmin and 2-methylisoborneol (2-MIB) produced by cyanobacteria, and these are typical off-flavor compounds (Fig. 1) [3]. The presence of metabolites and the by-products of cyanobacteria in water supply sources directly interferes with the quality of water, introducing negative effects by the attribution of taste and odor of water. The Ministry of Health, Labor and Welfare of the Japanese Government established "Drinking Water Quality Standards" for these musty and odorous compounds in 2004 [4]. Furthermore, several local waterwork bureaus have measured the musty odorous compounds for water quality monitoring in water resources and raw water [5–7].

In previous studies, cyanobacterial VOCs were detected from eight cyanobacterial strains including four different genera cultures. This provided interesting results such that β-ionone was commonly detected in a small amount in all cyanobacteria strains, whereas β-cyclocitral was predominantly produced by three *Microcystis* strains. β-Cyclocitral is also derived from β-carotene by an oxidation reaction [8]. They reported that the production of β-cyclocitral from β-carotene was activated by the disintegration of *Microcystis* cells using freezing and thawing. These results indicated that β-cyclocitral is not originally present in the cells and this can be derived from β-carotene when the *Microcystis* cell is disintegrated by stimulations, such as some pre-treatments and biological attacks by viruses and bacteria, and the subsequent activation of the carotenoid cleavage dioxygenases (CCD). β-Cyclocitral was more easily oxidized when compared to similar aldehyde compounds so that the pH of the solution quickly decreased to 4.5. An oxidation product of β-cyclocitral in the water solution was isolated and identified as 2,6,6-trimethylcyclohexene-1-carboxylic acid [9]. Under favorable conditions, β-cyclocitral may play a role in the lysis of the cyanobacteria in a natural environment and the blue color formation is considered as an indicator for the lysis of cyanobacteria by β-cyclocitral. Furthermore, the production of β-cyclocitral always accompanied the occurrence of 2-methyl-1-butanol and 3-methyl-1-butanol (Fig. 1). It was found for the first time that 2-methyl-1-butanol and 3-methyl-1-butanol were simultaneously produced by cyanobacteria and were excreted into the extracellular fraction. These alcohols are widely

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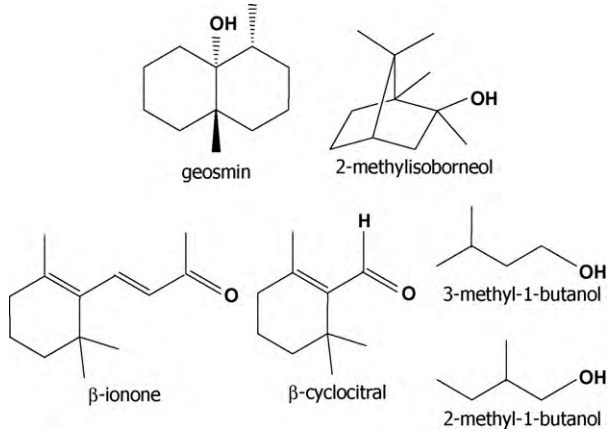

Fig. 1. Volatile organic compounds (VOCs) produced by cyanobacteria.

distributed in actinomyces [10], yeast [11], mold [12] and higher plants [13].

Thus, it was shown that the detailed VOCs production behavior by these cyanobacteria provided valuable information that can contribute to the prevention and control of taste and odor events in water sources. Additionally, these results suggest that VOCs from cyanobacteria may play a significant role in their ecological events. In order to fully understand the role of VOCs under natural conditions, it is essential to have an adaptable analytical method as the first step. Although conventional analysis methods have been devoted to geosmin and 2-MIB, few studies have been carried out for the VOCs other than the off-flavor compounds. In addition to these two compounds, β-ionone, β-cyclocitral, 2-methyl-1-butanol and 3-methyl-1-butanol were simultaneously analyzed together with 2,6,6-trimethylcyclohexene-1-carboxylic acid as an oxidation product of β-cyclocitral.

2. Experimental

2.1. Chemicals

The volatile compounds, β-cyclocitral, β-ionone, 2-methyl-1-butanol, 3-methyl-1-butanol, geosmin, and 2-methylisoborneol (2-MIB) standards were purchased from Wako Pure Chemical Industries (Osaka, Japan). Geosmin-d_3 from Hayashi Pure Chemical Industries (Osaka, Japan) was used as the internal standard for the GC/MS analysis. Silver oxide was obtained from Nacalai Tesque (Kyoto, Japan). HPLC grade methanol and formic acid from Wako Pure Chemical Industries and distilled water from Kanto Chemical (Tokyo, Japan) were used for the HPLC and LC/MS as the mobile phases.

2.2. Cyanobacteria

The cyanobacterial strains NIES-87 and NIES-298 belonging to *Microcystis* were obtained from the collection at the National Institute for Environmental Studies (NIES). These strains were cultured in Erlenmeyer flasks containing MA medium at 25 °C for 35 days under 28 μE/m^2 s continuous light and 100 rpm mixing or static conditions [14].

2.3. Simultaneous analysis of VOCs

Cultured broth or lake water (20 ml) was equally separated into two portions. After one was filtered through a GF/A glass filter (Whatman Japan, Tokyo, Japan), the resulting filtrate was used for the analysis of the dissolved VOCs and the other was used for the analysis of the total VOCs. These two portions were subjected to a head-space solid-phase microextraction (HS-SPME) coupled with GC/MS for the quantitative determination of the volatile compounds, β-cyclocitral, β-ionone, 2-methyl-1-butanol, 3-methyl-1-butanol, geosmin and 2-MIB. Briefly, the aliquot was placed in a head-space septum vial containing 4 g NaCl. The internal standard solution (10 μl) of geosmin-d_3 was added, the vial was sealed with a Teflon-lined cap and then gently agitated to dissolve the salt. After refrigerated stored for up to 24 h, the vial was placed in a stirrer equipped with a heater block preheated to 60 °C (Combi PAL system autosampler CTC Analytics, Zwingen, Switzerland). The outer needle of the SPME fiber 50/30 μm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethyl siloxane, Supelco, Bellefonte, PA, USA) assembly was passed through the septum and the fiber extended into the head-space. After 20 min, the fiber was retracted and placed in the injector of a gas chromatograph (Agilent 6890, Agilent Technologies, Palo Alto, CA, USA). The following operating conditions were then used: A J&W Scientific column DB-624 (30 m × 0.25 mm ID × 1.4 μm film), and a mass selective detector (Agilent 5973). Fiber desorption was performed in the splitless mode at 200 °C for 10 min. The injector temperature was 200 °C and the column temperature program was 40 °C (5 min), from 40 °C to 210 °C at 10 °C min^{-1} and a 5-min hold at 210 °C. Helium was used as the carrier gas (1 ml/min). The detector temperature was 230 °C. Electron ionization (EI) was used for the ionization and for the selected ion monitoring (SIM) mode; *m*/*z* 152 and 137 for β-cyclocitral; *m*/*z* 177 and 135 for β-ionone; *m*/*z* 70, 57 and 41 for 2-methyl-1-butanol; *m*/*z* 70, 55 and 41 for 3-methyl-1-butanol; *m*/*z* 112 and 111 for geosmin; *m*/*z* 95 and 107 for 2-MIB and *m*/*z* 115 and 114 for geosmin-d_3 were monitored.

2.4. Preparation of 2,6,6-trimethylcyclohexene-1-carboxylic acid from β-cyclocitral

β-Cyclocitral (0.95 g) was added to a solution of 2.5 mM silver oxide in 25 mM sodium hydroxide (10 ml), and the reaction mixture was allowed to stand for 3 days with stirring. The reaction mixture was acidified with 5% hydrochloric acid and extracted with diethyl ether. The extract was evaporated to give a residue, which contained a by-product in addition to the desired compounds. These were separated by recrystallization using benzene and cyclohexane to form the pure 2,6,6-trimethylcyclohexene-1-carboxylic acid (0.34 g).

2.5. GC/MS analysis for 2,6,6-trimethylcyclohexene-1-carboxylic acid in Lake Tsukui water

GC/MS was performed using a gas chromatograph (Agilent 6890N, 5973 inert), A J&W Scientific column DB-1MS (30 m × 0.25 mm ID × 0.25 μm film), and a mass selective detector (Agilent 5973). The sample (2 μl) was injected in the splitless mode. The injector temperature was 220 °C and the column temperature program was 40 °C (1 min), then from 40 °C to 180 °C at 8 °C min^{-1}. Helium was used as the carrier gas (0.6 ml/min). The detector temperature was 230 °C. Electron ionization (EI) was used at 70 eV for ionization and for the selected ion monitoring (SIM) mode; *m*/*z* 168, 153, 135 and 123 for 2,6,6-trimethylcyclohexene-1-carboxylic acid. The water collected from Lake Tsukui, Kanagawa, Japan, on August 5, 2008 was extracted with diethyl ether and the organic layer was dehydrated with sodium sulfate (anhydrous), and then injected into the same GC/MS.

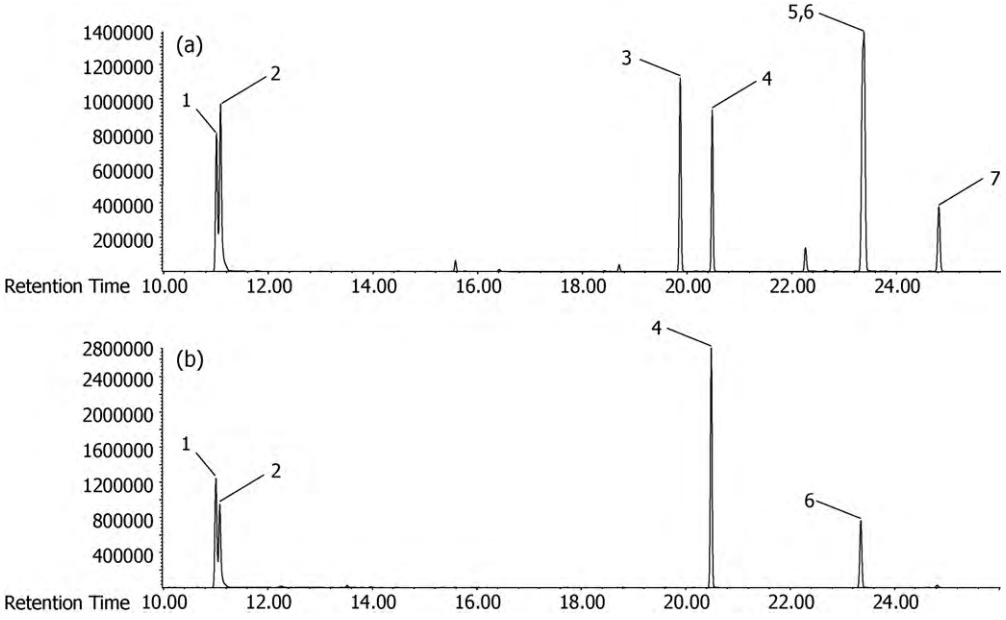

Fig. 2. Simultaneous analysis of VOCs: (a) standard VOCs and (b) VOCs from *Microcystis aeruginosa* NIES-87. Peak 1, 2-methyl-1-butanol; Peak 2, 3-methyl-1-butanol; Peak 3, 2-methylisoborneol (2-MIB); Peak 4, β-cyclocitral; Peak 5, geosmin; Peak 6, geosmin-d_3; Peak 7, β-ionone.

3. Results and discussion

3.1. Simultaneous analysis of volatile organic compounds produced by cyanobacteria

The collected VOCs were analyzed using DB-624 as the column under the stated operating conditions. The separated components were detected using a mass spectrometer under electron ionization (EI) conditions. Fig. 2(a) shows the total ion current (TIC) chromatogram of the standard 2 and 3-methyl-1-butanols, 2-MIB, β-cyclocitral, geosmin, geosmin-d_3, and β-ionone, and these peaks can be well separated except for the 2-methyl-1-butanol and 3-methyl-1-butanol under the optimized conditions. The following parameters were obtained by the experiments ($n = 5$) with standard samples: linear range, 0.2–100 μg/l for 2-methyl-1-butanol and 3-methyl-1-butanol and 0.005–10 μg/l for β-cyclocitral, β-ionone 2-methylisoborneol and geosmin; linearity, $r^2 > 0.999$ for β-cyclocitral, β-ionone and geosmin, $r^2 > 0.997$ for 3-methyl-1-butanol, $r^2 > 0.995$ for 2-methylisoborneol and 2-methyl-1-butanol; limits of quantification, 200 ng/l for each compound; limits of detection, 5 ng/l for β-cyclocitral, β-ionone and geosmin, 100 ng/l for 2-methyl-1-butanol and 3-methyl-1-butanol; relative standard deviations, >10% for β-cyclocitral, β-ionone, 3-methyl-1-butanol, 2-methylisoborneol and geosmin, 11.7% for 2-methyl-1-butanol. A typical TIC chromatogram of the VOCs from *Microcystis* sp. NIES-87 is shown in Fig. 2(b), in which 2-methyl-1-butanol, 3-methyl-1-butanol and β-cyclocitral are clearly detected. For the quantification, the selected ion monitoring (SIM) mode was employed using the characteristic fragment ions of each compound, and the results are now being reported. β-Cyclocitral, β-ionone and geosmin were almost present in the total fractions (data not shown). The total production of 2-methyl-1-butanol and 3-methyl-1-butanol by NIES-298 reached 1784 μg/l, and the latter was produced with one and half times the intensity compared to that of the former (Fig. 3). Furthermore, they were always present in the dissolved fraction.

There have been several analytical methods for VOCs from cyanobacteria [5–7,15,16] and we analyzed the VOCs in a culture broth and in lake water containing cyanobacteria using the modified method of Watson et al. [17]. Although the method was originally developed for trace amounts of VOCs in drinking water, it could be satisfactorily applied to our present study with proper dilution of the analyte solution. Therefore, 2-MIB, β-cyclocitral, geosmin and β-ionone could be simultaneously detected and quantified together with the 2- and 3-methyl-1-butanols (Fig. 2(a)). As described in Section 1, these alcohols were widely distributed in the environment. They are formed by deamination followed by decarboxylation of the branched-chain amino, acid such as L-leucine and L-isoleucine [18,19] or by way of intermediates from the branched-chain amino acid synthesis [20]. In the present study, these alcohols were simultaneously detected along with β-cyclocitral from the *Microcystis* strains (Fig. 2(b)). In previous studies, both alcohols of various origins were similarly analyzed by head-space SPME, but the separation between both alcohols was not attained [21] and not described in detail [22]. However, the proper use of the SIM enables both alcohols to be determined as shown in Fig. 2.

3.2. Analysis of 2,6,6-trimethylcyclohexene-1-carboxylic acid

During the investigation of β-cyclocitral from cyanobacteria, it was found that this compound is easily oxidized to yield the corresponding acid (2,6,6-trimethylcyclohexene-1-carboxylic acid),

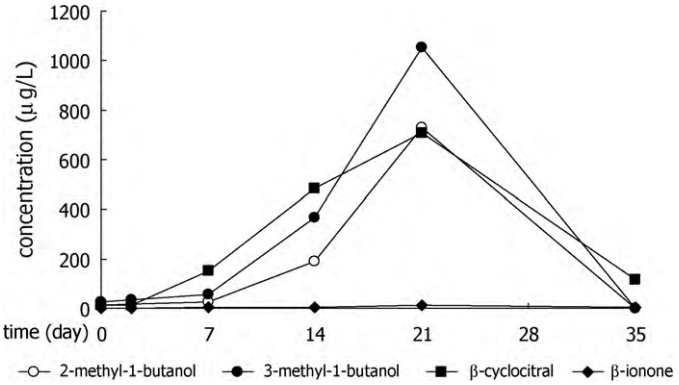

Fig. 3. Time course of the production of 2-methyl-1-butanol, 3-methyl-1-butanol, β-cyclocitral and β-ionone from *Microcystis aeruginosa* NIES-298.

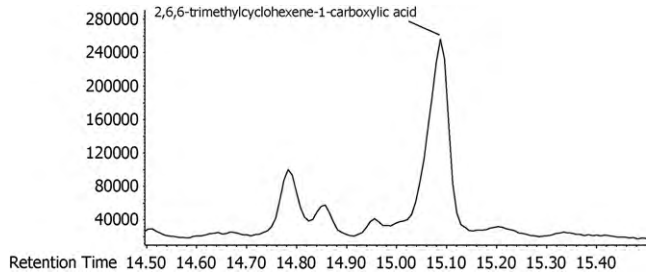

Fig. 4. Total ion chromatogram of the organic fraction collected from Lake Tsukui, in which the peak around 15 min is 2,6,6-trimet.hylcyclohexene-1-carboxylic acid.

which acidifies the surrounding area and causes the blue color formation of cyanobacteria [9]. Therefore, it was required to analyze the acid together with β-cyclocitral. The standard acid was obtained by the oxidation of β-cyclocitral with silver oxide. Because it was difficult to analyze it using the SPME method, it was analyzed under the usual conditions. In the obtained mass spectrum of 2,6,6-trimethylcyclohexene-1-carboxylic acid, the characteristic fragment ions, *m*/*z* 153 (base peak), 135, 123, 107 and 91, were observed together with the molecular ion at *m*/*z* 168. The SIM profile using the base peak was very effective, indicating that this compound can be sensitively detected by the usual GC/MS. The improved method was applied to a water sample containing cyanobacteria, which was collected from Lake Tsukui, Kanagawa, Japan, on August 5, 2008. Fig. 4 shows the TIC chromatogram of such a sample and the peak around 15 min is the desired acid. β-Cyclocitral provided the characteristic behavior, such that it lysed both the laboratory strains of any genera and bloom samples including many species of cyanobacteria and caused the characteristic color change from green to blue [9]. In the present study, it was confirmed that β-cyclocitral could be analyzed using the headspace SPME method and the corresponding acid using the usual GC/MS. These methods were successfully used to determine the reason for the occurrence of the blue color formation of cyanobacteria observed in a lake on August 5, 2008, and the obtained results will be reported elsewhere.

In conclusion, β-cyclocitral can lyse cyanobacteria in a natural environment and the lytic behavior is very characteristic [23]. The blue color formation due to acid stress is considered as an indicator of the lysis of cyanobacteria by β-cyclocitral [24]. Furthermore, the production of β-cyclocitral was always accompanied by the occurrence of 2-methyl-1-butanol and 3-methyl-1-butanol. 3-Methyl-1-butanol induced the formation of the hyphal-like extensions and pseudohyphae in yeasts [13]. As shown in this study, *Microcystis* produced large amounts of this compound together with 2-methyl-1-butanol in the death phase, suggesting that 3-methyl-1-butanol produced by only *Microcystis* has some functions associated with its life-cycle. These VOCs and related compounds may possibly play a significant role in their ecological events. In order to understand the function of these compounds, an analytical method was satisfactorily improved and these results using the method will contribute to the prevention and control of cyanobacterial events in water sources.

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