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Note

Determination of anatoxin-a, the neurotoxin of *Anabaena flos-aquae* cyanobacterium, in algae and water by gas chromatography–mass spectrometry

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Cyanobacteria, or blue-green algae, are known to include several potentially toxin-producing species. A toxic cyanobacterial bloom was first reported in 1878 to have caused deaths of domestic animals which had been drinking water containing bloom[1]. Toxic blooms have been verified in at least twenty countries thereafter, with several poisonings of both domestic and wild animals[2]. There are also indications that cyanobacterial toxins may pose a potential health risk for man. Sometimes they are even able to contaminate raw water sources; there are reports of cases where they have affected human health through domestic water use[3,4].

Several different types of toxins have been described[2]. Two of the most typical groups are hepatotoxic cyclic peptides and alkaloid neurotoxins. The only compound from the latter group that has been thoroughly characterized is anatoxin-a, or 2-acetyl-9-azabicyclo[4.2.1]non-2-ene (Fig. 1), produced by *Anabaena flos-aquae*. Toxic strains of *Anabaena* have previously been reported in Canada[5] and Finland[6].

Several methods for the determination of cyanobacterial hepatotoxins have been published[7–9]. Studies of methods for the determination of cyanobacterial neurotoxins has been less intensive; to my knowledge, only one report concerning the determination of anatoxin-a has been published[10].

The aim of this work was to develop a sensitive, simple, reliable and highly selective method for the determination of anatoxin-a in algae and in water and thus make it possible routinely to control potential hazard situations in raw water sources and recreational waters during *Anabaena* blooms. In order to obtain high sensitivity and selectivity, a low-resolution quadrupole mass spectrometer working in the selective ion monitoring (SIM) mode was used as a detector.

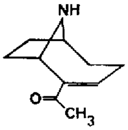

Fig. 1. Structure of anatoxin-a.

EXPERIMENTAL

Materials

The algal material was collected on August 19th, 1986, from a bloom in Lake Säyhteenjärvi, Artjärvi, southern Finland. The material gave typical symptoms of anatoxin-a poisoning when suspended in physiological sodium chloride solution and injected intraperitoneally into mice. Its anatoxin-a content was later shown to be 4.4 mg/g of lyophilized algal material[6].

Cyanobacteria used as blank samples were collected from Lake Pitkäjärvi, Espoo, southern Finland. The bloom consisted mainly of *Aphanizomenon* and was shown to be non-toxic by mouse bioassay.

The algal materials were freeze-dried in an Atlas pilot-scale freeze-dryer.

The organic solvents and other chemicals used were of analytical-reagent grade (Merck, Darmstadt, F.R.G.) or pesticide grade (Mallinckrodt, Paris, KY, U.S.A.). Kieselgel 60 plates (20 × 20 cm, 0.25 mm layer thickness; Merck) were used for thin-layer chromatographic (TLC) purification of anatoxin-a. Millex-HV filters (0.45 μm) and Sep-Pak silica cartridges (both from Millipore/Waters, Milford, MA, U.S.A.) were used in sample preparation.

Preparative purification of anatoxin-a

The preparation of anatoxin-a standards was performed by modifying the methods of Devlin *et al.*[5] and Abstrachan and Archer[10] in the following way. A 16.5-g amount of lyophilized algae was suspended in 1.5 l of distilled water and, after adjusting the pH to 5 with hydrochloric acid, the suspension was left to equilibrate overnight. It was then clarified by centrifuging at 10 000 rpm (9200 *g*) for 10 min and the clear solution was diluted to 2.5 l and adjusted to pH 10.5. The toxin was then concentrated into a 30 × 1.5 cm I.D. XAD-2 column, which had first been washed consecutively with distilled water, acetone and 0.01 *M* sodium carbonate solution. The dilute toxin solution was poured slowly through the column, which was then washed with 2 l of sodium carbonate solution. The toxin was eluted with 200 ml of 0.01 *M* hydrochloric acid–ethanol solution which was then evaporated in a rotary evaporator to *ca.* 15 ml. A similar volume of distilled water was added and the solution was washed with two 30-ml volumes of *n*-hexane. After adjusting the pH to 11 with sodium hydroxide solution, anatoxin-a was extracted four times with 40 ml of chloroform and then back-extracted from the combined chloroform solutions with three 50 ml-volumes of 0.001 *M* hydrochloric acid. The yellowish solid material obtained by drying the solution was further purified by TLC with methanol–chloroform (1:4) as eluent. The zone of R_F = 0.2–0.4 containing anatoxin-a was scraped off and boiled in acidic methanol under reflux for 2 h. Anatoxin-a was again extracted from the alcohol solution as described previously and the dried material (*ca.* 20 mg) used for the analytical standards.

Method for determining anatoxin-a in algal material

The algae are freeze-dried. The leafy material thus formed is homogenized and mixed thoroughly and an aliquot of 20 mg placed in a centrifuge tube for analysis. A 10-ml volume of methanol and 0.1 ml of concentrated hydrochloric acid are added and the suspension is mixed thoroughly and extracted in an ultrasonic bath for 15 min. The suspension is centrifuged, the extraction step repeated and the combined solutions filtered through a Millex-HV filter. The solution is then evaporated nearly to dryness in a Kuderna–Danish evaporator. The residue is dissolved in 1 ml of chloroform, 1 ml of concentrated acetic acid and 0.5 ml of acetic anhydride are added, the solution is mixed gently and the test-tube closed tightly. The mixture is then left to react overnight (16 h) at 50°C. The excess of reagent and the solvent are again evaporated nearly to dryness in a Kuderna–Danish evaporator (no smell of acetic acid should be detectable) and the residue is dissolved in 1 ml of chloroform. The solution is transferred into a Sep-Pak silica cartridge, which is cleaned with 7.5 ml of acetone–chloroform (5:95). Acetylanatoxin-a is finally eluted with 2 ml of methanol, which is then concentrated under a gentle stream of nitrogen to about 100 μl and chloroform is added to a final volume of 1 ml. This solution is used for gas chromatographic–mass spectrometric (GC–MS) analysis.

Method for determining anatoxin-a in water

A 12-ml volume of 0.25 *M* sodium carbonate solution is added to 100 ml of water and the solution is mixed thoroughly. It is then extracted three times with 100 ml of chloroform and the combined chloroform solutions are concentrated to about 10 ml in a rotary evaporator. A 5-ml volume of methanol and 0.1 ml of concentrated hydrochloric acid are added and the solution is mixed thoroughly and concentrated nearly to dryness in a Kuderna–Danish evaporator. The derivatization step is per-

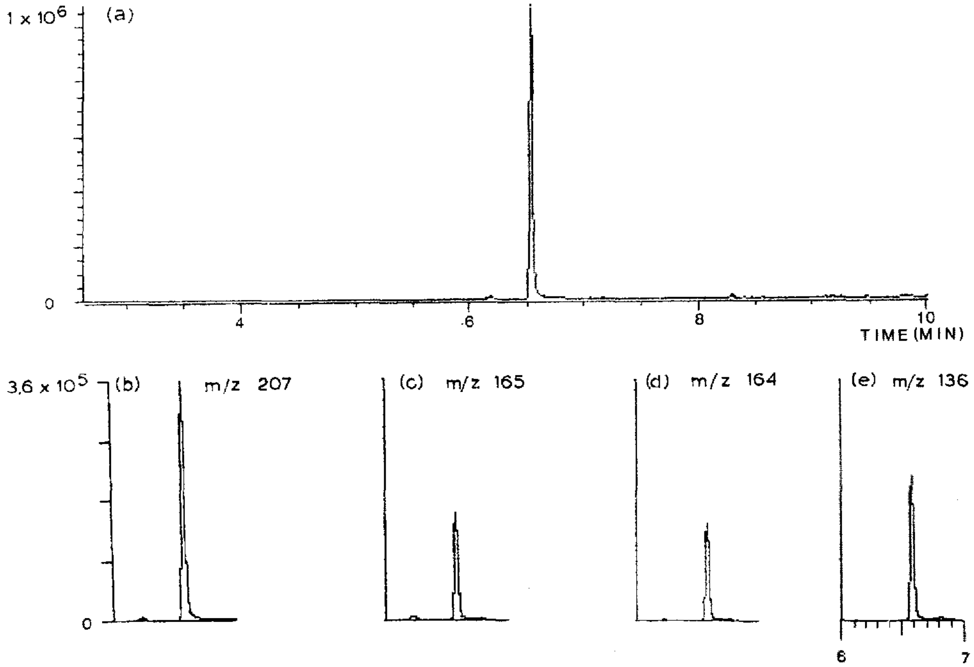

Fig. 2. Typical GC–MS traces from a run with an *Anabaena flos-aquae* sample. Top, total ion chromatogram; below, ion chromatograms with four monitored ions.

formed as above, although with relatively clean waters the Sep-Pak purification is normally not necessary.

GC–MS analysis

A GC–MS system consisting of a Hewlett-Packard 5790 gas chromatograph, an HP 5970B mass-selective detector and an HP 59970C ChemStation data system was used in the determination step. The column was 15 m × 0.2 mm I.D. with HP-1 cross-linked methylsilicone as stationary phase with a 0.33-μm film thickness (Hewlett-Packard, Avondale, PA, U.S.A.). Helium was used as the carrier gas at an injector pressure of 0.5 kg/cm^2 and the oven was temperature programmed from 80 to 275°C at 15°C/min. The volume injected was 1 μl, splitless time 45 s and the injector temperature 240°C. Ions of m/z = 207, 165, 164 and 136 were used for monitoring anatoxin-a. A typical group of ion chromatograms is presented in Fig. 2.

RESULTS AND DISCUSSION

The adequacy of the extraction step was checked by doubling the solvent volumes and by performing a second extraction from samples that had already been treated. The linearity of the method for anatoxin-a in algal material was tested by mixing various amounts of dried *Anabaena* and non-toxic *Aphanizomenon*. The anatoxin-a contents of these mixtures were determined in triplicate. Fig. 3 shows the detector signal as a function of amount of anatoxin-a injected; the test runs were performed with samples containing 1–20 mg of *Anabaena* in a 20-mg sample. The concentration range was selected to cover the most typical concentrations of anatoxin-a in Finnish *Anabaena* blooms[6]. The limit of determination (LOD) lies far below these concentrations at the level of 0.1 ng of anatoxin-a injected assuming a signal-to-

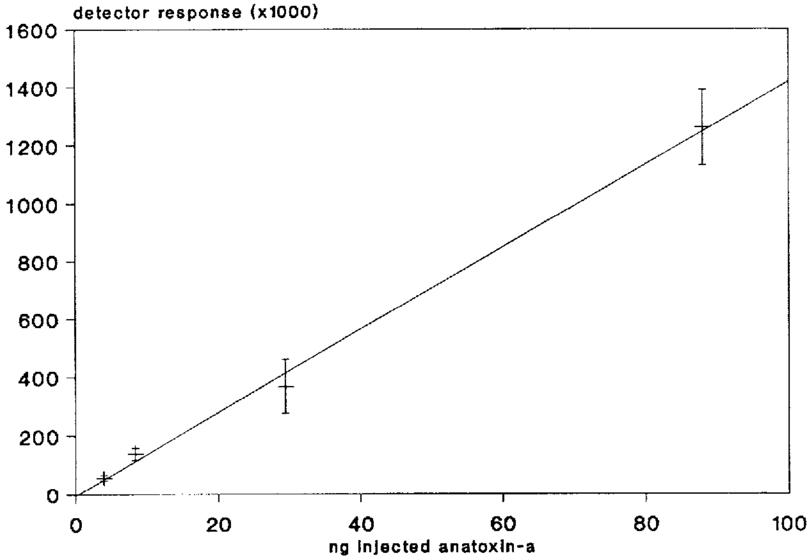

Fig. 3. Detector response (m/z 207) as a function of amount of anatoxin-a injected. Average of triplicate determinations ± S.D.

noise ratio of 100:1 at *m/z* 207, which is considered satisfactory regarding the natural background from the samples. The LOD corresponds to a concentration of 5 μg/g in dry algal material, an extremely low value for typical blooms, or 1 μg/l in water.

Cases in which anatoxin-a has to be determined in water samples are rare. In natural waters it should only be present in connection with an *Anabaena* bloom and then the algae themselves can be examined. However, if a bloom exists in a raw water source and the water treatment procedure does not include an effective filtration step, there is a possibility of finding anatoxin-a in the treated water also[11]. It is in such a situation that a method is needed for determining anatoxin-a in water.

The methods described above have been used in this laboratory for routine determinations of anatoxin-a in samples of algal blooms and in investigations concerning the behaviour of anatoxin-a in commonly used raw water treatment procedures, the results of which will be published elsewhere[11]. They have proved to fulfil the original aims of simplicity, selectivity and sensitivity and therefore it is expected that they could fill a need also in other laboratories concerned with research on the aqueous environment.

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