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# Analysis of anatoxin-a in aqueous samples by solid-phase microextraction coupled to high-performance liquid chromatography with fluorescence detection and on-fiber derivatization

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**Abstract**

A simple method for analysis of anatoxin-a in aqueous samples was developed using solid-phase microextraction (SPME) and high-performance liquid chromatography (HPLC) with fluorescence detection. Anatoxin-a was derivatized to a fluorogenic agent on the surface of the SPME fiber. In the method an SPME fiber was immersed for 30 min in the aqueous sample. The fluorogenic derivatizing reagent (4-fluoro-7-nitro-2,1,3-benzoxadiazole, 1.0 mg/ml in methanol) was dropped or sprayed onto the fiber containing extracted analytes. The fiber was then heated for 10 min in an empty vial at 70 °C in a waterbath to promote derivatization. The derivatives formed on the fiber were desorbed in a SPME–HPLC interface. The interface was filled with methanol–1 m*M* hydrochloric acid (7:3, v/v) before inserting of the fiber into the interface. For desorption, the fiber was inserted in the interface for 5 min. For anatoxin-a in an aqueous sample, the calibration curve showed linearity in the range of 50–1500 ng/ml and the limit of detection of anatoxin-a was 20 ng/ml. No interferences were found, and the time for analysis was 55 min for one sample. 

*Keywords:* Solid-phase microextraction; Anatoxins; Toxins

## 1. Introduction

Toxic cyanobacteria have been discovered in lakes and drinking water in many countries [1]. It is well known that cyanobacteria produce cyclic peptide hepatotoxins (microcystins, nodularins) and neurotoxins [anatoxin-a, homoanatoxin-a, anatoxin-a(s)]. These toxins present acute and chronic hazards to human and animal health and are responsible for isolated, sporadic animal fatalities (mammals, fish, birds) each year [2]. It is important to routinely monitor the levels of these toxins in reservoirs and drinking waters where cyanobacterial blooms occur, because human health problems are associated with the ingestion of, and contact with cyanobacterial blooms and their toxins.

Anatoxin-a (2-acetyl-9-azabicyclo[4.2.1]non-2-ene) is a neurotoxin alkaloid which is produced by a number of blue-green algae (cyanobacteria) including *Anabaena* spp., *Aphanizomenon* spp. and *Oscillatoria* spp. [3]. The current interest in anatoxin-a relates to increasing reports of animal death following algal bloom ingestion and to toxicological aspects of drinking water quality. Several studies have examined the levels of anatoxin-a in reservoir and drinking water following the appearance of algal blooms [4–12]. The aim of this work was to develop

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selective, sensitive, and solvent-free methods for the analysis of anatoxin-a in aqueous samples.

Previous studies indicated that the analysis of anatoxins required a large volume of water sample. In order to analyze low concentrations of anatoxin-a in aqueous samples, purification and evaporation of anatoxin-a from aqueous samples were required. In addition, derivatization was needed to detect the anatoxins with HPLC or GC prior to analysis because anatoxins have no specific UV absorbance to identify them. Therefore, the sample preparation procedures described in the previous reports were laborious and time consuming. A precise, simple and rapid method for the quantitative analysis of anatoxin-a is required in environmental or toxicological practices.

Solid-phase microextraction (SPME) involves the partitioning of analytes between a polymer coated fiber and sample matrix, and integrates sampling, extraction, concentration and sample introduction [13]. Many applications have been reported to analyze organic and inorganic compounds in environmental and biological materials using GC, HPLC and CE [14]. Some derivatization methods have been applied to alter the properties of analytes before or after the SPME procedure. Derivatization has the advantage of altering the chemical characteristics of compounds. For example, the polar compounds are converted to the nonpolar compounds, which can be detected by gas chromatography [15–17].

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) reacts selectively with primary and secondary amines to form high fluorescence derivatives [18]. NBD-F is a convenient and useful reagent because the derivatization occurs in a few minutes in an aqueous system such as a borate buffer. In this study, the derivatization–SPME technique has been applied to the extraction of anatoxin-a in aqueous samples. For this purpose, the derivatization of anatoxin-a with NBD-F was performed on the SPME fiber after extraction of analytes.

## 2. Experimental

### 2.1. Materials

Anatoxin-a fumarate was purchased from Sigma (Mississauga, Canada). NBD-F was purchased from Fluka. The structure of anatoxin-a and reaction scheme are shown in Fig. 1. Other reagents and solvents used were purchased at the highest commercial quality. All aqueous solutions were prepared using deionized water (NANOpure, Ultrapure water system). The stock standard solution (1.0 mg/ml) of anatoxin-a was dissolved in water and stored at 4 °C in a refrigerator.

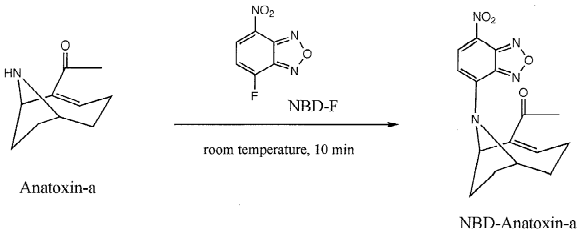


Fig. 1. Structural formula of anatoxin-a and reaction scheme for the derivatization of anatoxin-a with NBD-F.

A manual SPME assembly and replaceable extraction fibers, coated with polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane–divinylbenzene (PDMS–DVB, 60 μm), polyacrylate (PA, 85 μm), and Carbowax–temprated resin (CW–TPR, 50 μm) were purchased from Supelco (Canada). The fibers were washed with mobile phase for 30 min prior to use.

### 2.2. High-performance liquid chromatography

The HPLC system used was a Tosohaas 6040 pump (distributed by Supelco) and a Varian 9070 fluorescence detector (Varian Associates, Canada), equipped with a 50×4.6 mm I.D. reversed-phase column (Restek, Ultra $C_{18}$, 5 μm). Methanol–water (60:40, v/v) was used as the mobile phase. The mobile phase was filtered using a filter (0.45 μm), and degassed by using vacuum followed by sonication. The flow-rate was 0.5 ml/min. The column temperature was ambient (~22 °C). The excitation and emission wavelengths were set at 495 and 530 nm, respectively. Data were collected and integrated with a personal computer using Varian STAR 4.5 software.

### 2.3. SPME–HPLC interface

The assembly of the SPME–HPLC interface is

similar to that described previously [19]. In brief, the interface includes a custom-designed desorption chamber and a six-port Rheodyne 7161 injection valve. When the injection valve is at the 'load' position, the desorption chamber is at ambient pressure so that the fiber can be placed into the chamber. When the injection valve is turned to the 'injection' position, the eluent flow desorbs the analytes from the fiber onto the HPLC column in a very short period.

### 2.4. Sample preparation for analysis

A spiked sample (1.6 ml) and sodium chloride (320 mg) were placed into a 2-ml amber vial, and sealed rapidly with a silicone septum and a screw cap. The needle of the SPME device containing an extraction fiber was inserted through the septum of the vial and the extraction fiber was immersed into the sample. During extraction, the sample was agitated at 1000 rpm using a stirring bar. After 30 min, the needle was removed from the vial and the derivatization reagent (1.0 mg/ml in methanol) was placed on the surface of the coating. When the reagent was placed on the coating, two different dropping procedures (microsyringe and spray) were used and compared. After the evaporation of the droplet solvent, the needle was inserted into the empty vial and heated at 70 °C for 10 min. After reaction, the needle was inserted in the SPME–HPLC interface. The interface was filled with desorption solution before inserting of the fiber into the interface. The derivatives on the fiber were desorbed by exposing the fiber for 5 min in the interface.

### 2.5. Microsyringe procedure

A microsyringe was used for placing the derivatizing reagent on the fiber. The derivatizing reagent (3 μl) was withdrawn by a microsyringe and placed onto the fiber coating. After evaporation of the droplet solvent, the fiber was transferred to the heated empty vial for the reaction to take place.

### 2.6. Spray procedure

The sprayer used was a spray type dispenser. The derivatizing reagent was filled into the spray vial (4.0 ml). The fiber was sprayed in a fume hood. The reagent was sprayed onto the fiber and the fiber was then transferred to the heated empty vial for the reaction to take place.

### 2.7. Optimal condition of reaction and extraction

Four different coatings were examined in this study to extract anatoxin-a (1000 ng/ml) from the spiked buffer (10 m*M*, pH 10). Each fiber was exposed in water sample for 10 min. The fiber was then heated at 60 °C for 10 min. The CW–TPR fiber coating provided higher extraction yields than the other fiber coatings in this study.

The effect of reaction temperature and time on the derivative of anatoxin-a with CW–TPR from an aqueous sample was examined. An aqueous sample containing 1000 ng/ml of anatoxin-a was prepared. The fiber was exposed for different periods at three different temperatures (room temperature, 60 and 70 °C) and then analyzed.

To determine the effect of extraction time on the adsorbed amount of anatoxin-a, the fiber was exposed in the water sample for six different periods of time (2, 5, 10, 20, 30 and 60 min). The fiber was then heated at 70 °C for 10 min.

### 2.8. Method validation

To examine calibration curve of anatoxin-a in an aqueous sample, the samples spiked with anatoxin-a at concentrations ranging from 10 to 2000 ng/ml were prepared and analyzed using the above procedure. The calibration curve was obtained by plotting the peak area against the concentration of anatoxin-a. Reproducibility was evaluated by analyzing aqueous samples containing two different concentrations (100 and 1000 ng/ml) of anatoxin-a on the same day in five replicates.

## 3. Results and discussion

### 3.1. Coating selection

SPME is an equilibrium process where analytes are transferred from sample matrices to a fiber coating [13]. The extracted analytes are then directly desorbed from the fiber into the desorption solution in the SPME–HPLC interface. The amount of analytes extracted by the fiber coating is dependent on

the partition coefficient or affinity of the analytes toward the fiber coatings. It is necessary to select a suitable fiber coating that provides a high enough recovery of analytes for good detection sensitivity. Four different types of coating were examined to extract anatoxin-a from water samples. The fibers were conditioned as described in the experimental section and tested with mobile phase flow stream to demonstrate that there were no interfere compounds in the fibers. The amount extracted from the CW–TPR fiber coating was higher than that of the other fiber coatings for anatoxin-a (Fig. 2). Therefore, the CW–TPR fiber coating was selected and used for the subsequent experiments. Generally, polar compounds are more likely to be adsorbed on polar coatings [13,20]. The CW–TPR fiber coating provided higher extraction yields than the other fibers in this study.

### *3.2. Desorption process*

The desorption procedure is important in HPLC and needs to be optimized for each application as well as for different solvent compositions [21]. In order to select an optimal desorption solvent, the ratio of methanol and hydrochloric acid (1 m*M*) were varied and tested. The fiber was inserted in the interface for 5 or 10 min to desorb the anatoxin-a derivative into the desorption solvent.

Increasing the proportion of methanol in the desorption solvent decreased the peak area of the derivative. When hydrochloric acid (1 m*M*) only was used for the desorption solution, the peak shape was becoming broad because the analyte desorption from the fiber was very slow. When using methanol–hydrochloric acid (7:3, v/v), a sharp and symmetrical peak of the anatoxin-a derivative was obtained and the peak area increased. The peak shape and the peak area of the anatoxin-a derivative were not improved with an increase in the desorption time. Therefore, methanol–hydrochloric acid (7:3, v/v) was selected for the desorption solvent, and the fiber was desorbed in the interface for 5 min.

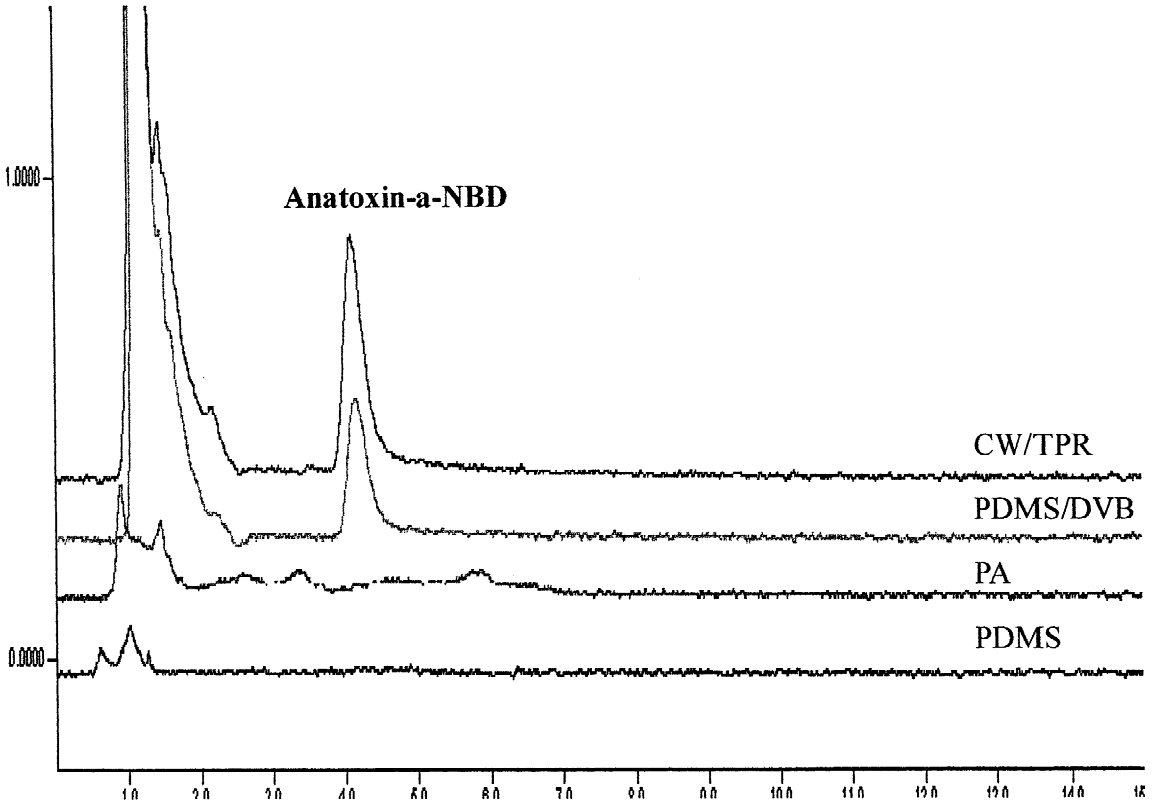


Fig. 2. HPLC chromatogram of the anatoxin-a derivative obtained from aqueous samples spiked with the different coatings.

Table 1
Effect of reaction temperature and time on the derivatization yields of anatoxin-a in the aqueous sample

| Reaction temperature | Peak area of NBD-anatoxin-a Reaction time (min) | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 5 | 10 | 20 | 30 | 60 |
| Room temp. | | 1 046 256 | 1 836 123 | 2 684 216 | 3 125 537 | 3 025 437 |
| 60 °C | 3 002 527 | 3 983 426 | 4 497 351 | 5 429 657 | 5 253 519 | 5 359 632 |
| 70 °C | 3 395 665 | 4 295 863 | 2 269 955 | 6 000 536 | 5 962 357 | 5 998 883 |

The sample concentration was 1000 ng/ml. Extraction vials contained spiked samples (pH 10). Samples were stirred at 1000 rpm; extraction was by CW–TPR (50 μm) at room temperature for 30 min. The reaction was performed at the temperature and time indicated. Separation and detection were as described in the Experimental section.

### *3.3. Reaction temperature and time with derivative reagent*

The effect of reaction temperature and time of anatoxin-a with NBD-F is shown in Table 1. The derivatizing reagent and anatoxin-a were nonvolatile compounds. To accelerate the reaction of the derivatizing reagent and anatoxin-a, the fiber was heated in an empty vial. The anatoxin-a derivative formed increased as the temperature increased. The amount of anatoxin-a derivative formed was maximized at 70 °C. Therefore, a temperature of 70 °C was adopted for subsequent experiments.

The reaction of anatoxin-a and NBD-F is promoted by eliminating hydrogen fluoride (HF) from the vial because the reaction kinetics is affected by HF concentration. After extraction, the fiber was heated in a vial containing triethylamine, which trapped hydrogen fluoride. However, triethylamine affected the chromatogram and the baseline was overlapped with the peak of the anatoxin-a derivative. The limit of detection with triethylamine however was higher than that without triethylamine.

### *3.4. Salting-out effect*

The salting-out effect has been described in several methods [22]. Extraction recovery is increased with the addition of a salt to sample matrices when salt effectively decreases the solubility of analytes. A salt, such as sodium chloride, is commonly used to improve an extraction recovery.

The effect of addition of sodium chloride to a sample is shown in Table 2. The amount of anatoxin-a extracted increased with the concentration of sodium chloride. The maximum amount of anatoxin-a was found at the salt concentration of 20% (w/v) and slightly decreased with a higher salt concentration. The concentration of sodium chloride used in subsequent experiments was 20%.

### *3.5. Extraction time*

The effect of extraction time on the adsorbed amount of anatoxin-a is shown in Table 3. The adsorbed amount of anatoxin-a was maximized at 30 min and decreased slightly with longer extraction times. The exposure time of 30 min was used, because the exposure time of 30 min gave sufficient amount of derivative formed to be analyzed and had a small RSD. One water sample could be analyzed in 55 min, including HPLC analysis time of 15 min.

An increase in sampling temperature will decrease equilibrium analyte recovery [13]. When the sampling temperature is higher than the maximum adsorption temperature, the partition coefficient of the analytes decreases and the fiber coating loses its

Table 2
Effect of NaCl concentration on the derivatization yields of anatoxin-a in the aqueous sample

| | NaCl concentration (%) | | | | |
|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 35 |
| Peak area | 2 861 346 | 2 953 467 | 4 439 276 | 6 100 392 | 8 761 936 |

The sample concentration is 1000 ng/ml. Conditions were as for Fig. 3 except for the following; reaction was at 70 °C for 10 min.

Table 3
Extraction time profile for the derivatization yields of anatoxin-a in the aqueous sample

| | Extraction time (min) | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 5 | 10 | 20 | 30 | 60 |
| Peak area | 834 529 | 1 455 395 | 3 259 642 | 5 264 235 | 6 258 555 | 6 523 638 |

The sample concentration was 1000 ng/ml. Conditions were as for Fig. 3 except for the following: reaction was at 70 °C for 10 min.

ability to absorb analytes. Therefore, this sampling was done at room temperature.

### *3.6. Calibration curve, limit of detection, and repeatability*

The typical HPLC chromatogram of the anatoxin-a derivative from the spiked water sample is shown in Fig. 3. A sharp and clear peak of the anatoxin-a derivative was obtained without disturbance of endogenous interferences. The retention time variation of the anatoxin-a derivative was not depended on concentration, and the standard deviation did not exceed 0.02 min (Table 4). The intra-day RSD values in peak area were 7.6% (average). Considering the RSDs, the proposed method was found to be reproducible compared with other SPME results.

HPLC chromatograms of the anatoxin-a derivative obtained from aqueous samples spiked with different concentration of anatoxin-a (100, 200, 500 and 1000 ng/ml) are shown in Fig. 4. The calibration curve showed linearity in the range of 50–1500 ng/ml for anatoxin-a by the two different derivatization methods (Table 5). The correlation coefficients of the calibration curve was 0.996. The method limit of detection was 20 ng/ml ($S/N = 3$). In previous reports [7], concentrations of anatoxin-a in water were found to be 2–444 ng/ml. Although a very low concentration of anatoxin-a is not monitored by this proposed method, anatoxin-a can be detectable for

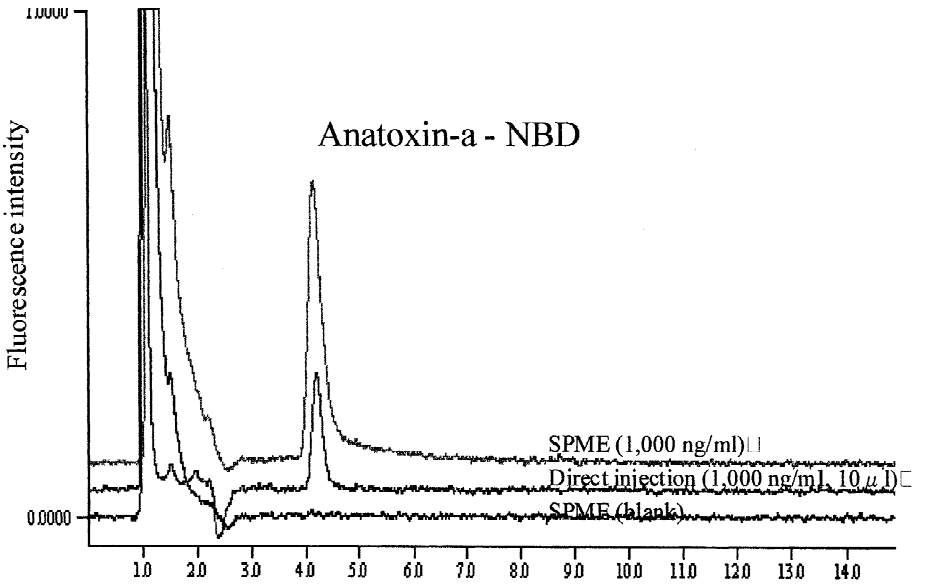


Fig. 3. Typical HPLC chromatogram of the anatoxin-a derivative obtained from a spiked sample (1000 ng/ml) using (a) the CW–TPR fiber and (b) a direct injection, and HPLC chromatogram obtained from (c) a blank sample using the CW–TPR fiber. For the direct injection, 100 μl of an anatoxin-a spiked sample (1000 ng/ml) was reacted with 50 μl of NBD-F (1 mg/ml, in methanol). A 50-μl volume of hydrogen chloride (0.1 *M*) was added to the reaction mixture to quench the reaction. The mixture was diluted with the mobile phase (200 μl) and then 40 μl of the sample was injected to HPLC.

Table 4
Repeatability of retention time and peak area of the anatoxin-a derivative

| Anatoxin-a | Retention time | | Peak area | |
|---|---|---|---|---|
| concentration | mean (min) | RSD (%) | mean (counts) | RSD (%) |
| Micro-syringe | | | | |
| 100 ng/ml | 4.165 | 0.75 | 656 210 | 5.4 |
| 1000 ng/ml | 4.143 | 0.75 | 7 014 670 | 7.9 |
| Spray | | | | |
| 100 ng/ml | 4.167 | 0.61 | 729 996 | 8.2 |
| 1000 ng/ml | 4.173 | 0.79 | 6 032 324 | 7.7 |

Experiments were performed in five replicates.

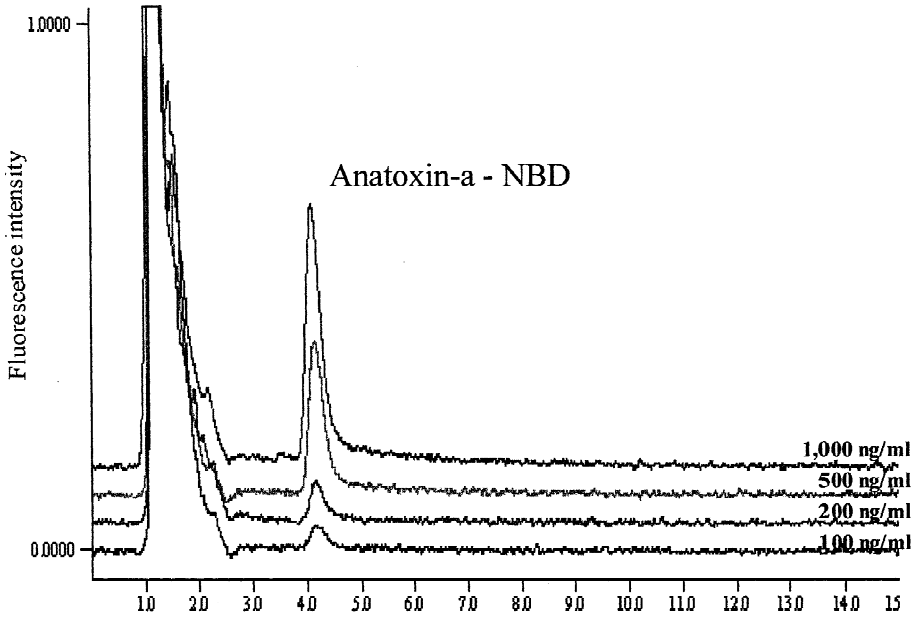


Fig. 4. HPLC chromatogram of the anatoxin-a derivative obtained from aqueous samples spiked with different concentrations of anatoxin-a (100, 200, 500 and 1000 ng/ml) extracted with the CW–TPR fiber as described previously.

Table 5
Quantitation limit and linearity of the method

| Procedure | Limit of detection (ng/ml) | Range of linearity (ng/ml) | Linearity[a] | Correlation coefficient |
|---|---|---|---|---|
| Micro-syringe | 20 | 50–1500 | $y = 7154.3x + 133645$ | 0.999 |
| Spray | 20 | 50–1500 | $y = 6184.6x + 113838$ | 0.997 |

[a] $x$ = Amount of analyte (ng/ml), and $y$ = peak area.

highly contaminated samples using this SPME method.

### *3.7. Application for monitoring of drinking water*

This proposed method was applied to analysis of lake and drinking water. Anatoxin-a was not found in any samples. As samples were collected in winter, cyanobacteria growth was not expected. Anatoxin-a was detected from the lake sample into which anatoxin-a was spiked, and no interference occurred from the matrix. This method can be applied for monitoring and screening anatoxin-a from seriously contaminated water and cultured medium of Cyanobacteria.

## 4. Conclusion

The potential of SPME–HPLC–fluorescence detection for analysis of anatoxin-a in aqueous samples by on-fiber derivatization with NBD-F has been demonstrated. The CW–TPR fiber was found to be the optimal coating for extraction of anatoxin-a. Derivatization with NBD-F was performed on the SPME fiber coating after extraction of anatoxin-a from aqueous sample. Two methods were demonstrated for addition of the derivatizing reagent to the coating: one was a microsyringe procedure, the other was a spray procedure. A good repeatability (RSD$<$ 10%) was obtained by the both procedures. For desorption of the derivatives in the interface, the interface was filled with methanol–1 m*M* hydrochloric acid (7:3, v/v) before inserting of the fiber into the interface. The calibration curve showed linearity in the range of 50–1500 ng/ml for anatoxin-a in an aqueous sample. The limit of detection of anatoxin-a was 20 ng/ml in an aqueous sample. This method can be applied to monitoring and screening anatoxin-a in highly contaminated water and cultured medium of Cyanobacteria.

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## References

[1] G.A. Codd, T.M. Jefferies, C.W. Keevil, E. Potter, Detection Methods For Cyanobacterial Toxins, Royal Society of Chemistry, Cambridge, 1994.
[2] G.A. Codd, C.J. Ward, S.G. Bell, Arch. Toxicol. Suppl. 19 (1997) 399.
[3] W.W. Carmichael, J. Appl. Bact. 72 (1992) 445.
[4] K.J. James, I.R. Sherlock, Biomed. Chromatogr. 10 (1996) 46.
[5] K.J. James, I.R. Sherlock, M.A. Stack, Toxicon 35 (1997) 963.
[6] M. Takino, S. Daishima, K. Yamaguchi, J. Chromatogr. A 862 (1999) 191.
[7] K. Minberg, J. Chromatogr 481 (1989) 358.
[8] K. Harada, Y. Kimura, K. Ogawa, M. Sizuki, A.M. Dahlem, V.R. Beasley, W.W. Carmichael, Toxicon 27 (1989) 1289.
[9] D.K. Stevens, R.I. Krieger, J. Anal. Toxicol. 12 (1988) 126.
[10] K.J. James, A. Furey, I.R. Sherlock, M.A. Stack, M. Twohig, F.B. Caudwell, O.M. Skulberg, J. Chromatogr. A 798 (1998) 147.
[11] C. Bumke-Vogt, W. Mailahn, W. Rotard, I. Chorus, Phycologia 35 (1996) 51.
[12] R. Draisci, E. Ferretti, L. Palleschi, C. Marchiafava, Food Addit. Contam. 18 (2001) 525.
[13] J. Pawliszyn, Solid Phase Microextraction: Theory and Practice, Wiley, New York, 1997.
[14] J. Pawliszyn (Ed.), Application of Solid-phase Microextraction, Royal Society of Chemistry, Cambridge, 1998.
[15] L. Pan, J. Pawliszyn, Anal. Chem. 69 (1997) 196.
[16] P.A. Martos, J. Pawliszyn, Anal. Chem. 70 (1998) 2311.
[17] P.D. Okeyo, N.H. Snow, J. Microcol. Sep 10 (1998) 551.
[18] K. Blau, J.M. Halket (Eds.), Handbook of Derivatives For Chromatography, Wiley, Chichester, New York, 1993.
[19] A.A. Boyd-Boland, J. Pawliszyn, Anal. Chem. 68 (1998) 1521.
[20] V. Mani, in: Properties of Commercial Spme Coatings: Application of Solid-phase Microextraction, Royal Society of Chemistry, Cambridge, 1998, p. 57.
[21] Y.-C. Wu, S.-D. Huang, Anal. Chem. 71 (1999) 310.
[22] H. Lord, J. Pawliszyn, J. Chromatogr. A 902 (2000) 17.