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Analysis of anatoxin-a using polyaniline as a sorbent in solid-phase microextraction coupled to gas chromatography–mass spectrometry

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Abstract

A simple and sensitive method for determining anatoxin-a in aqueous samples was developed using solid-phase microextraction (SPME) and gas chromatography with mass spectrometry (GC–MS) detection. Three forms of polyaniline (PANI) films and a single form of polypyrrole (PPY) film were prepared and applied for SPME. The extraction properties of these films to anatoxin-a were examined and it was shown that leucoemeraldine form of PANI displayed a better selectivity to this compound. SPME conditions were optimized by selecting the appropriate extraction parameters, including type of coating (leucoemeraldine form of PANI at 32 μm thicknesses), salt concentration (10%, w/v), time of extraction (30 min) and stirring rate (1000 rpm). The calibration curve was linear in the range from 50 to 10 000 ng/ml, with the detection limit ($S/N = 3$) of 11.2 ng/ml. This method was successfully applied for the analysis of anatoxin-a in the cultured media of two species of cyanobacteria.

Keywords: Anatoxin-a; Solid-phase microextraction; Polyaniline; Polypyrrole; Leucoemeraldine form

1. Introduction

The occurrence of toxic cyanobacterial blooms and scums is a world-wide phenomenon, with instances found in over 30 countries. It has been investigated in fresh, brackish or marine waters throughout Europe, North and South America, Australia, Africa and Asia [1].

The toxins of cyanobacteria can be grouped into three categories: hepatotoxins (microcystins, nodularins and cylindrospermopsin), neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a(s) and saxitoxins) and lipopolysaccharide endotoxins [2].

Anatoxin-a (2-acetyl-9-aza bicyclo [4.2.1] non-2-ene) (Fig. 1) is a secondary amine alkaloid, produced by species of Anabaena and Cylindrospermum, as well as, Aphanizomenon flos-aquae and benthic members of the Oscillatoria/Phormidium group [3]. Anatoxin-a has a high toxicity (LD_{50} i.p.mouse 200–250 μg/kg) [4] and it is a neuromuscular blocking agent which acts as a postsynaptic cholinergic, nicotinic agonist [5].

Anatoxin-a degrades readily, especially in sunlight and at high pH, to non-toxic degradation products [8]. Two important degradation products of anatoxin-a are epoxy and dihydroanatoxin-a which have been identified from various blooms [6].

A number of analysis methods have been proposed for anatoxin-a, such as; gas chromatography (GC) with electron capture detection (ECD) following derivatization [7], gas chromatography–mass spectrometery (GC–MS) [8], high performance liquid chromatography (HPLC) with ultra-violet [9,10] and fluoresence [6,11] detection of its

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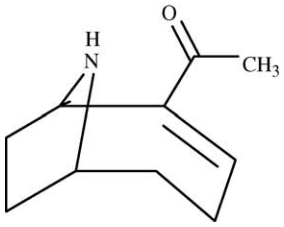

Fig. 1. Structure of anatoxin-a.

derivatives and various methods using mass spectrometric (MS) detection [3,12–16]. Recently, Pawliszyn et al. introduced a new method for analysis of anatoxin-a in aqueous samples by SPME-HPLC with fluoresence detection [17].

Polyaniline (PANI) exists in a variety of protonation and oxidation forms: Leucoemeraldine (fully reduced form) (Fig. 2a), emeraldine (Fig. 2b) and pernigraniline (fully oxidized form) (Fig. 2c) [18]. Polypyrrole (PPY) exists in a doped form (Fig. 2d) with electrolyte anions as dopant agents [19].

To date, several experimental coatings in SPME have been prepared and investigated for a range of applications. In addition to commercial coatings such as polydimethylsiloxane (PDMS) for general applications, other more specialized materials such as polymeric coatings have been developed. The widely used polymers in SPME are based on polypyrrole (PPY), polythiophene and polyaniline (PANI). Of these three classes of materials, PPY and its derivatives have been mostly used and intensively studied in recent years [20].

In this study, coatings consist of gold wires which are electrochemically coated with PANI and PPY were used as sorbent agents in SPME and analyses were carried out by GC–MS. The coated surfaces of PANI and PPY were studied by scanning electron microscopy (SEM). The results show that leucoemeraldine form of PANI is a very good sorbent for extraction of anatoxin-a at low concentration level (11.2 ng/ml), with high linearity (50–10 000 ng/ml) and low relative standard deviation (2.7%).

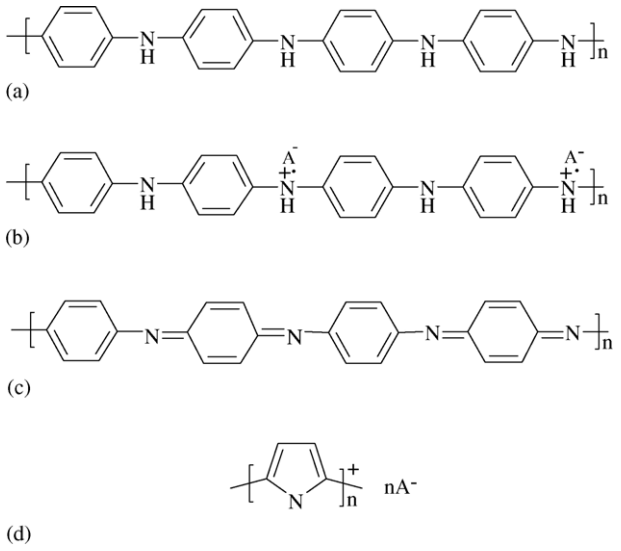

Fig. 2. Structure of three forms of coated PANI: leucoemeraldine form (a), emeraldine form (b), pernigraniline form (c) and PPY coated (d).

2. Experimental

2.1. Reagents

Anatoxin-a fumarate was purchased from Sigma (Mississauga, Canada). Aniline (99.55%) and pyrrole (98%) were purchased from Aldrich (Mississauga, Canada) and distillated before use. NaCl, Na_2CO_3 and $NaHCO_3$ salts, methanol (HPLC grade), hydrochloric acid (≥32%) and sulfuric acid (≥98%) were purchased from Merck (Darmstadt, Germany). All aqueous solutions were prepared using deionized water. Deionized water was prepared using a Milli-Q (Millipore, Bedford, MA, USA) purification system. The stock standard solution (1.0 mg/ml) of anatoxin-a was dissolved in deionized water and stored at 4 °C in a refrigerator.

Cultured species of cyanobacteria were obtained from Caspian See Bony Fishes Research Center (Gilan, Iran). The algae tested in this study were *Nostoc carneum* (*Fremy and Geitler*) and *anabaena SP2*.[1]

2.2. Instrumentation

A manual SPME assembly was purchased from Azar Electrod (Urmia, Iran). The Snigders Scientific (Tilburg, The Netherlands) freeze-dryer was used for freeze-drying of algae material at −50 °C and 0.08 mbar. Electropolymerization was performed using a Metrohm 746 VA Trace Analyzer (Herisau, Switzerland), which was controlled by electrochemical software VA Data Base2. A Universal centrifuge from Rad Producting Co. (Tehran, Iran) was used for centerifuging of solutions. For scanning electron microscopy (SEM), PANI- and PPY-coated wires, covered with a gold film and then analyzed using a Philips XL30 scanning electron microscope (The Netherlands) at 25 kV accelerating potential. A magnetic stirrer MR 3001 K from Heidolph (Germany) and a magnetic stirring bar in cylindrical shape with ring from Witeg (Germany) were used for stirring the samples during extraction.

A Varian 3800cp GC equipped with a flame ionization detector with a cp-sil 5CB column, (30 m × 0.25 mm I.D., film thickness 0.25 μm contained 100% polydimethyl siloxane), (Chrompack, USA) was used for all of optimization procedures. The oven was temperature programmed as follows: 40 °C (1 min) to 250 °C (15 min) at 15 °C/min. The carrier gas was nitrogen (purity 99.999%) at a flow-rate of 1.5 ml/min. The volume injected for obtaining *F* (detector response factor) (See Eq. (1) in Section 3.1) was 1 μl, the mode of injection was splitless during 45 s and then the carrier gas was split with

[1] This species is maintained in Caspian See Bony Fishes Research Center of Iran.

split ratio (1:50). The injector and flame ionization detector temperature were 250 and 280 °C, respectively. Data were acquired with a Varian Star system. A Fisons 8000 gas chromatograph coupled with a mass detector Trio 1000, equipped with a capillary column cp-sil 5CB, 30 m × 0.25 mm I.D., 0.25 μm film thickness, containing 100% polydimethylsiloxane (Chrompack, USA) was used under the same chromatographic conditions of GC except that helium was used as carrier gas. The interface was mainted at 200 °C and the spectra were obtained at 70 eV.

2.3. Preparation of PANI and PPY films

PANI and PPY films were prepared electrochemically using a three-electrode system. The reference electrode was Ag/AgCl (Metrohm, 3 M KCl). The counter electrode was a platinum wire in cylindrical shape with 1.8 mm O.D. and 2 cm length (Metrohm), and a gold wire (250 μm O.D., 1.5 cm length) from Azar Electrod Co. was used as a working electrode. All potentials applied were relative to the corresponding reference [22,23]. All the polymeric films were prepared at ambient temperature (25 °C). The solutions were deoxygenated by bubbling nitrogen for 200 s prior to use. The PANI films were generated from solution of distillated aniline (0.1 M) in deionized water (Milli-Q, Millipore), using H_2SO_4 (1.0 M) electrolyte. Films were deposited by cyclic voltammetry. Pernigraniline form of PANI was produced by sweeping continuously between −0.2 and +1.1 V (versus Ag/AgCl) after six cycling. For the preparation of leucoemeraldine form, fully reduced PANI form, after sweeping the potential from −0.2 to +1.1 V (versus Ag/AgCl), the potential was stopped at −0.2 V (versus Ag/AgCl) during 600 s. For the formation of emeraldine (salt) form of PANI, sweeping the potential (from −0.2 V) was stopped at +0.335 V (versus Ag/AgCl).

The PPY film was prepared from an aqueous solution of distillated pyrrole (0.1 M) using H_2SO_4 (0.2 M) electrolyte. The film was prepared by six cycles between 0.00 and +0.8 V (versus Ag/AgCl) at a scan rate of 10 mV/s. All prepared films were washed with methanol and dried with nitrogen. According to the SEM study, the estimated thicknesses of all prepared films were 32 μm.

2.4. Preparation of standard solutions

Anatoxin-a can be stored as supplied (fumarate salt) at −20 °C for up to 1 year and stoke solutions at −20 °C for up to 3 months. 2 ml sodium carbonate buffer solution (10 mM, pH 10) was spiked with 2 μl stock standard solution and was placed in a 4-ml dark vial in which sodium chloride (200 mg) was added and rapidly sealed with a silicone septum and a screw cap. For sampling, the fiber is first withdrawn into the syringe needle, which is used to penetrate the septum of sample vial. The fiber is then inserted into the sample solution by pushing the plunger. The fiber is completely immersed into the sample solution. The sample solution in the vial is stirred with a magnetic stirring bar. The speed of rotation of stirring bar was 1000 rpm, extraction time was 30 min and the temperature of sample solution was 25 ± 2 °C. After extraction, the plunger was withdrawn to retract the fiber into the needle and syringe needle was removed from the vial. For desorption, the needle was inserted into the GC injection port and then the fiber was exposed to the hot injector of GC system for analysis. Thermal desorbtion of anatoxin-a was carried out for 5 min. Analyte carry over (memory) effect was not observed because the concomitants were desorbed completely by the carrier gas flow during 5 min.

2.5. Preparation of real samples for analysis

Freeze-dried cyanobacterial material (1 g) was extracted by stirring with methanol (100 ml) contaning hydrochloric acid (1 M, 1 ml) for 10 h. The solution was centrifuged at 5000 rpm for 10 min and the supernatant was collected. The pellet was re-extracted twice and the combined extracts were evaporated to dryness at 50 °C under nitrogen. The residues

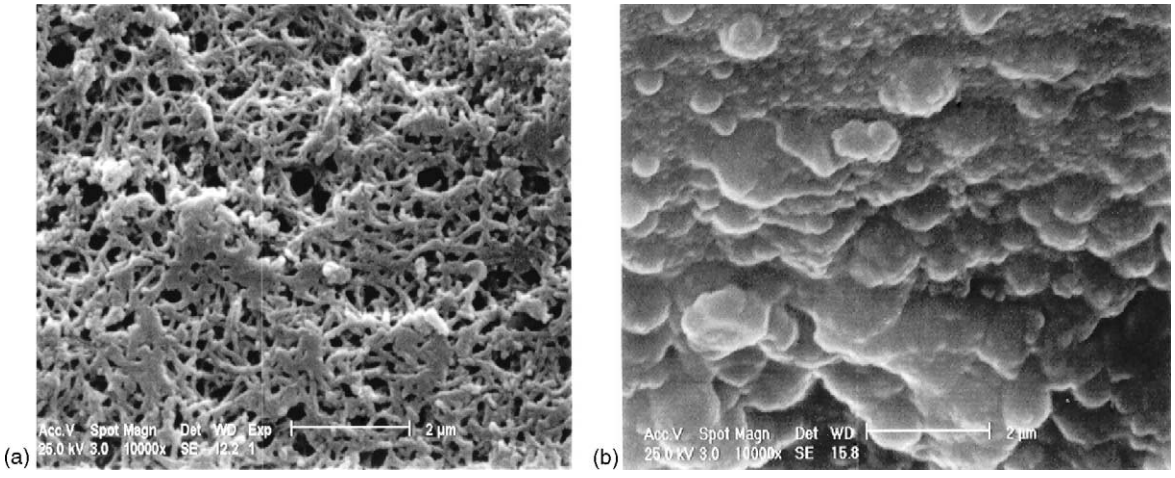

Fig. 3. Scanning electron micrograph of the leucoemeraldine-PANI (a) and PPY (b) coated gold wires surface at 10000× magnifications.

were reconstituted with 15 ml deionized water and the SPME prodecure was carried out.

3. Results and discussion

3.1. Optimization of SPME conditions

Four parameters were optimized to achieve the best extraction efficiency for SPME. These parameters include the type of coating, concentration of salt, extraction time profile and speed of stirring. All parameters were evaluated on the basis of extraction yield (%) which are the percentage of extracted amount of analyte (n_A) per initial amount of analyte in a 2-ml sample solution. The amount of analyte extracted was calculated as:

$$n_A = FA = \left(\frac{m}{A_d}\right) A \quad (1)$$

where n_A is the amount (mass) of analyte extracted by SPME, F is the detector response factor which can be calculated by comparing the amount of analyte (m) injected and the area counts (A_d) obtained by liquid injection ($F = m/A_d$) and A is the response (area counts) obtained by SPME. The results are discussed below.

All optimization procedures were carried out by GC-FID, and each analysis was repeated for three times.

3.1.1. Coating selection

In the present study, four coatings (leucoemeraldine form of PANI, emeraldine salt form of PANI, pernigraniline form of PANI and doped form of PPY) were evaluated for their extraction efficiencies to the anatoxin-a. The extraction yield (%) values for analyte concentration of 1000 ng/ml were 0.26 for leucoemeraldine, 0.22 for emeraldine salt, 0.084 for pernigraniline and 0.14 for doped PPY. Among them leucoemeraldine form of PANI shows higher extraction yield (%) than other coatings for anatoxin-a. Therefore, the leucoemeraldine form of PANI coating was selected and used for the subsequent experiments. Extraction periods of 30 min were chosen for all coatings since it was equivalent to run the GC chromatogram and samples can be analyzed continuously.

The outputs from the sensing units depend on the nature of the polymeric films. The extraction ability of polymeric films is due to the intractions between polymer and analyte, such as base–acid, dipole–dipole interactions and hydrogen bonding, in addition to the π–π and hydrophobic intractions. The increased affinity of PANI and PPY to anatoxin-a can be attributed to π–π intraction and hydrogen bonding between PANI and PPY polymers and the compound.

The PANI- and PPY-doped and undoped sorbents showed different effects to adsorbtion of the compound. The difference in extraction efficiencies can be due to the ability of hydrogen bounding formation between polymers and the compound, since in buffer solution with pH 10, positively charged anatoxin-a salt (fumarate salt) neutralized, can form hydrogen bonding with leucoemeraldine form of PANI, which has free NH groups, better than PPY and emeraldine salt forms of PANI, which their has radical cation NH groups. The results showed that the extraction yield (%) is decreased when there is a decrease in the free NH groups. Pernigraniline, which is fully oxidized, has a lowest ability to extract anatoxin-a, while emeraldine form of PANI and PPY with some non-oxidized NH groups have higher affinity toward anatoxin-a than leucoemeraldine PANI form. Generally, it can be seen that PANI forms have a higher affinity toward anatoxin-a than PPY. The SEM results demonstrated these observations. As shown in Fig. 3, PANI has fibrous structure, while PPY has globular structure. The surface of PANI is much smoother than that of PPY film, and its porosity is more than PPY.

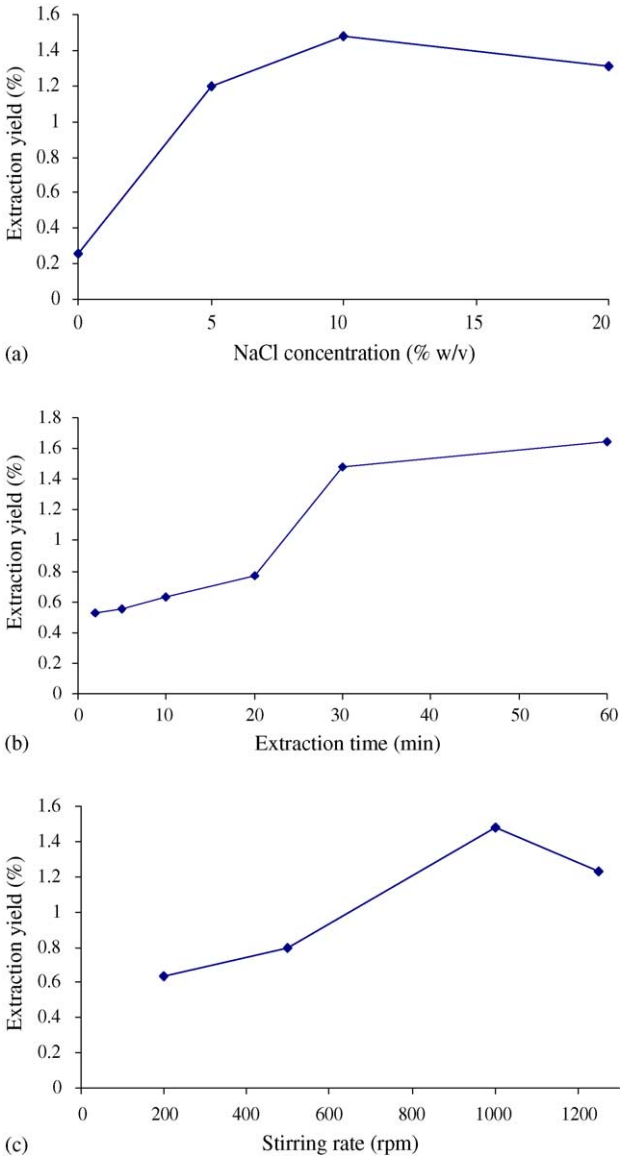

Fig. 4. Optimization of SPME procedure: effect of NaCl concentration (a), effect of extraction time (b) and effect of stirring rate (c) on extraction yield. Analyte concentration in the sample: 1000 ng/ml.

It is known, porous structures should significantly increase the effective surface areas of the films and therefore, higher extraction efficiency can be expected compared with non-porous films, so, the higher extraction efficiency of PANI films than PPY, can be attributed to their surface morphology. It seems, in extraction process of pernigraniline form of PANI and PPY, hydrogen bonding is more effective than porosity.

3.1.2. Salting-out effect

Extraction recovery is increased with addition of salt to sample matrices as salt effectively reduces the solubility of

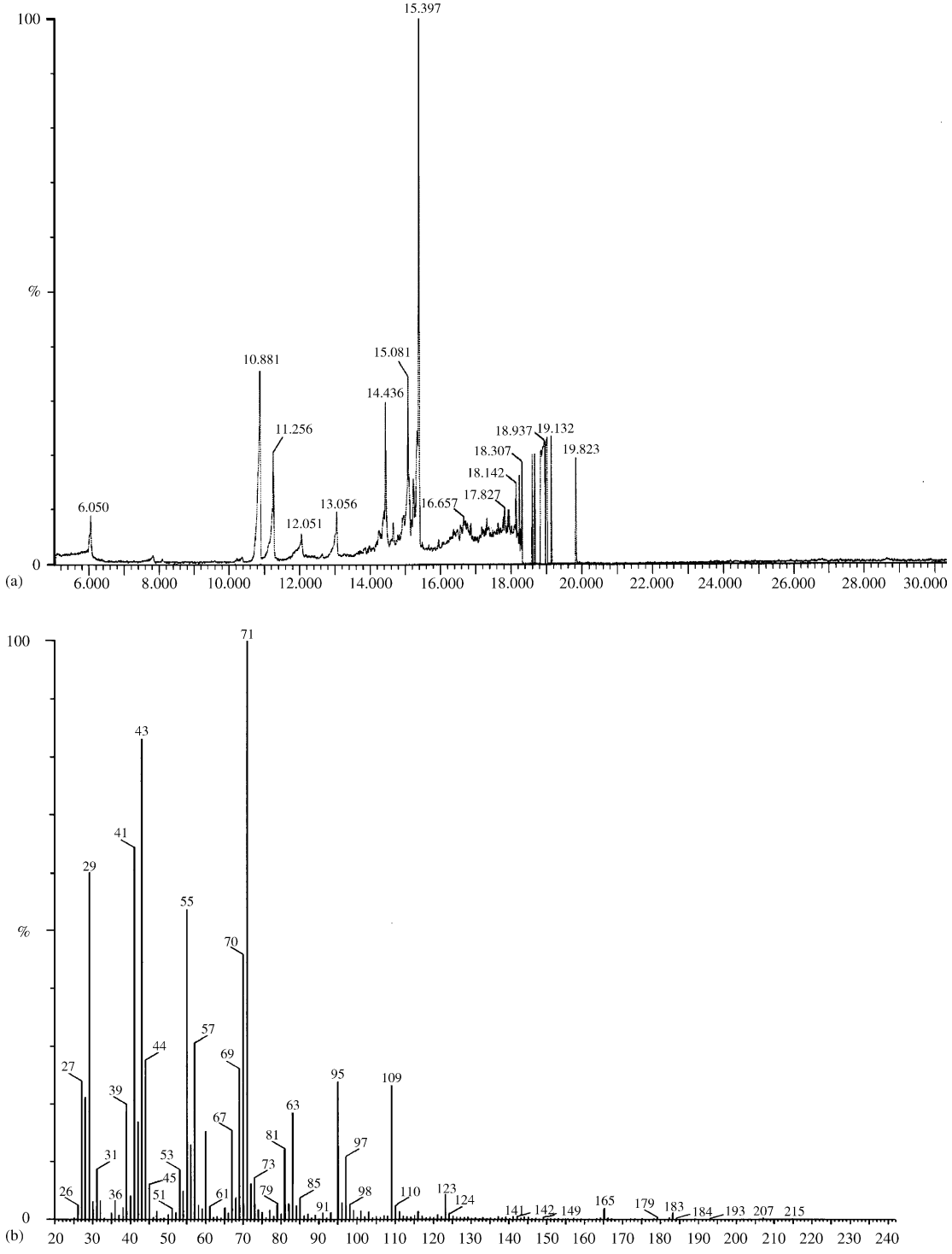

Fig. 5. Total ion current (a) and mass spectrum (b) obtained by SPME-GC–MS of real sample solution (*Nostoc carneum*).

analytes. The effect of addition of sodium chloride to samples is shown in Fig. 4a. The results show that 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 10% (w/v) and decreased slightly with higher salt concentration. Therefore, the concentration of sodium chloride used in subsequent experiments was 10% (w/v).

3.1.3. Extraction time

The effect of extraction time on the adsorbed amount of anatoxin-a is tested. As shown in Fig. 4b, the amount of analyte extracted increased when the time of extraction increases from 2 to 30 min. After 30 min, the amount of analyte extracted continue to increase but with smaller slopes. However, a further increase in the extraction time increases the analysis time, which is not desirable for the routine analysis.

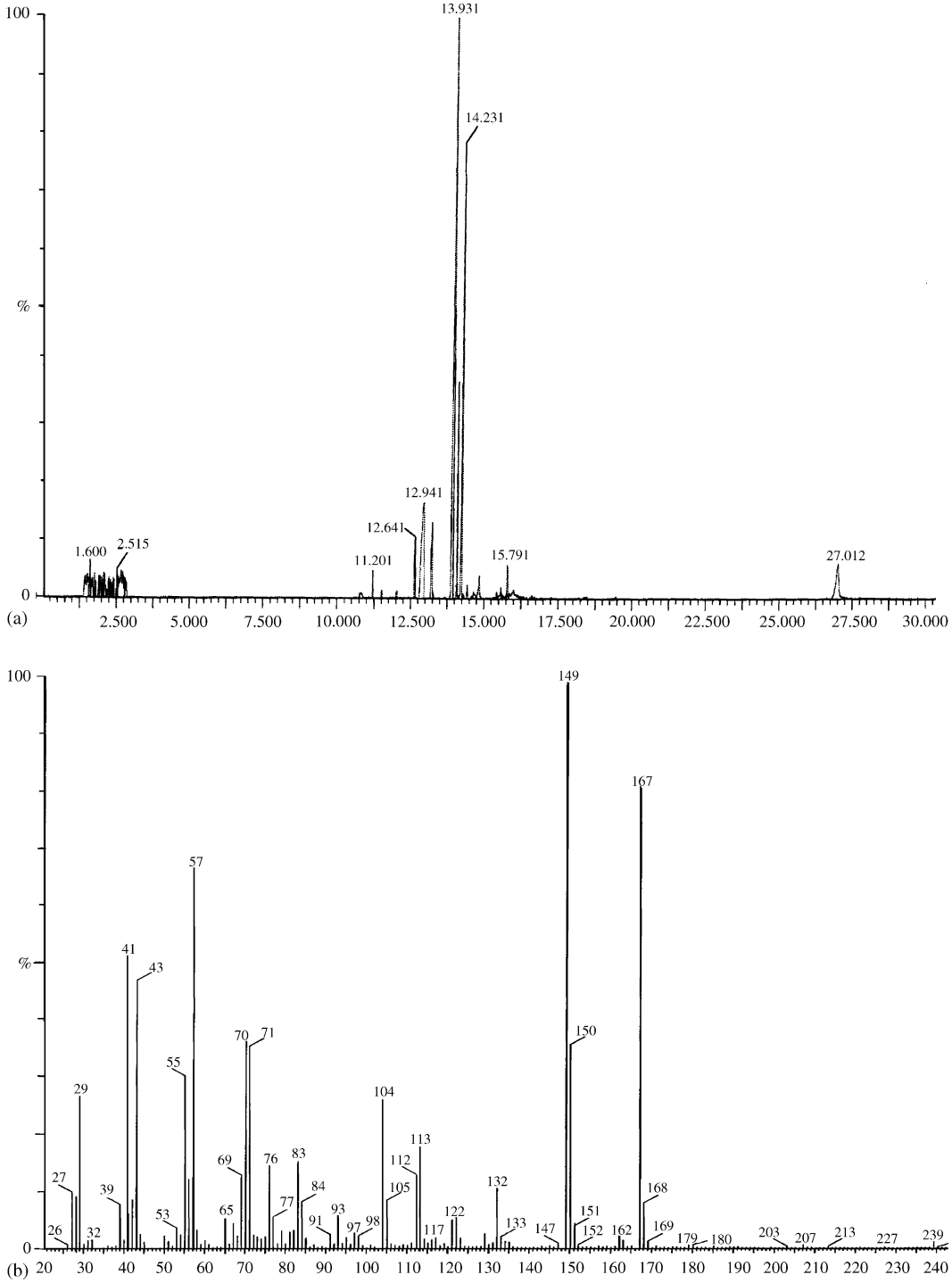

Fig. 6. Total ion current (a) and mass spectrum (b) obtained by SPME-GC–MS of real sample solution (*Nostoc carneum*) after 1 day of sample preparation.

3.1.4. Speed of stirring

The efficiency of the extraction may be increased by stirring of the aqueous solution and it is important to maintain a constant stirring speed in order to obtain reproducible results. PANI film was exposed to standard solution (1000 ng/ml) for 30 min at different stirring speeds. The sample solution (2 ml) was stirred in a 4-ml vial by magnetic stirring bar with 3 mm O.D. and 8 mm length. The effect of stirring rate is shown in Fig. 4c. Maximum amount of adsorbed anatoxin-a was obtained at 1000 rpm.

3.2. GC–MS analysis of anatoxin-a standard solution

The analysis of standard sample showed the retention time 11.241 min for anatoxin-a with conditions given in the experimental section. The mass spectrum showed fragments of 165, 150, 136, 122, 94, 68 and 43 *m*/*z* that related to anatoxin-a. After exposing the sample to light and passing one day of sample preparation, the peak in 11.241 min was disappeared and a new peak in 27.072 min was observed that according to its molecular ion (at *m*/*z* 167) and similar fragments to anatoxin-a in its mass spectrum, it can be attributed to a related compound of anatoxin-a.

3.3. GC–MS analysis of anatoxin-a in cyanobacterial cells

The proposed method was applied to analysis of two cultured species of cyanobacteria. Both of real samples, the total ion currents confirmed the results obtained from standard solution. TIC and MS spectra of one of real samples (*Nostoc carneum*) are shown in Fig. 5. The Peak in 11.256 min is related to anatoxin-a. After exposing the sample to light and passing one day of sample preparation, result as same as standard solution was seen for the real samples. As shown in Fig. 6 peak in 11.256 min disappeared and a new peak in 27.012 min was observed. Quantification of the samples was carried out by peak area using the external standard calibration. The obtained results were 0.156 and 0.248 mg/g lyophilized cells for *Nostoc carneum* (*Fremy and Geitler*) and *anabaena SP2*, respectively. The concentration of anatoxin-a was the average value of the three measurements. This method can also be applied for monitoring and screening anatoxin-a from seriously contaminated water.

3.4. Precision, limit of detection and linearity

The calibration curve showed linearity in the range of 50–10000 ng/ml for anatoxin-a and linear regression equation was $y = 5.0783x + 344.58$ where x is amount of analyte (ng/ml) and y is the peak area. The correlation coeffcient of the calibration curve was 0.9997. Method limit of detection ($S/N = 3$) and quantitation were 11.2 and 200.1 ng/ml, respectively. In this work, by precisely controlling the film preparation conditions and performing SPME under the same conditions, the differences between films were low. Table 1 shows repeatability of retention time and peak area of anatoxin-a. The retention-time variation of anatoxin-a was not dependent on concentration.

Table 1
Repeatability of retention time and peak area of anatoxin-a

Anatoxin-a concentration (ng/ml)	Retention time		Peak area	
	Mean (min)	RSD (%)	Mean (counts)	RSD (%)
100	11.51	0.65	44197	4.8
1000	11.19	0.64	466492	5.5

Experiments were performed in three replicates.

The intra- and inter-day precision of the method were investigated by analyzing of three samples with same concentration and each analysis was repeated for three times. The same determination was performed for 3 days (three times each day). The RSD (%) of intra-day and inter-day assay were 5.15, 2.7, respectively.

4. Conclusions

A simple and practical GC and GC–MS method in combination with SPME for the determination of anatoxin-a in cultured medium of cyanobacteria and contaminated water is presented. Also the ability and potential application of the PANI and PPY coatings for extraction of anatoxin-a from algae matrices have been demonstrated. Maximum response was obtained using a leucoemeraldine base form of PANI film, a 30 min extraction time, 10% (w/v) salt concentration and 1000 rpm stirring rate.

The results of using PANI films for SPME are in good agreement with the result expected from the structures of the polymers and with those obtained by this procedure. The calibration curve showed linearity in the range of 50–10 000 ng/ml for anatoxin-a in aqueose sample. The limit of detection of anatoxin-a was 11.2 ng/ml for measurement in the aqueous sample.

References

[1] S.G. Bell, G.A. Codd, Agricultural Chemicals and the Enviroment, Royal Society of Chemistry, 1996, p. 109.
[2] K. Sivonen, G. Jones, Cyanobacterial toxins, in: I. Chorus, J. Bartram (Eds.), Toxic Cyanobacteria in Water—A Guide to their Public Health Consequence, Monitoring and Management, World Health Organization, E & FN Spon, Routledge, London, 1999, p. 41.
[3] C. Edwards, K.A. Beattie, C.M. Scrimgeour, G.A. Codd, Toxicon 30 (1992) 1165.
[4] D.K. Stevens, R.I. Krieger, Toxicon 29 (1991) 167.
[5] W.W. Carmichael, J. Appl. Bacteriol. 72 (1992) 445.
[6] K.J. James, A. Furey, I.R. Sherlock, M.A. Stack, J. Chromatogr. A 798 (1998) 147.
[7] D.K. Stevens, R.I. Krieger, J. Anal. Toxicol. 12 (1998) 126.
[8] K. Himberg, J. Chromatogr. 481 (1989) 358.
[9] A. Zotou, T.M. Jefferies, Analyst 118 (1993) 753.
[10] M.W. Powell, Chromatographia 45 (1997) 25.
[11] K.J. James, I.R. Sherlock, Biomed. Chromatogr. 10 (1996) 46.

[12] M.M. Ross, D.A. Kidwell, J.H. Callahan, J. Anal. Toxicol. 13 (1989) 317.
[13] G.K. Poon, L.J. Griggs, C. Edwards, K.A. Beattie, G.A. Codd, J. Chromatogr. 628 (1993) 215.
[14] K.-I. Harada, H. Nagai, Y. Kimura, M. Suzuki, H.D. Park, M.F. Watanabe, R. Luukkainen, K. Sivonen, W.W. Carmichael, Tetrahedron 49 (1993) 9251.
[15] M. Takino, S. Danishima, K. Yamaguchi, J. Chromatogr. A 862 (1999) 191.
[16] A. Furey, J. Crowley, M. Lehane, K.J. James, Rapid Commun. Mass Spectrom. 17 (2003) 583.
[17] A. Namera, A. So, J. Pawliszyn, J. Chromatogr. A 963 (2002) 295.
[18] W.S. Huang, B.D. Humphrey, A.G. Mac Diarmid, J. Chem. Soc. Faraday Trans. 1 82 (1986) 2385.
[19] J. Wu, J. Pawliszyn, J. Chromatogr. A 909 (2001) 37.
[20] H. Lord, J. Pawliszyn, J. Chromatogr. A 885 (2000) 153.
[22] D.T. Hoa, T.N. Suresh Kumar, N.S. Punekar, R.S. Srinivasa, R. Lal, A.Q. Contractor, Anal. Chem. 64 (1992) 2645.
[23] D.N. Upadhyay, S. Bharathi, V. Yegnaraman, G.P. Rao, Solar Energy Mater. Solar Cells 37 (1995) 307.