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VISUAL DETECTION OF CYANOBACTERIAL TOXINS BY THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

This paper reports the detection of microcystins by non-instrumental visual detection using thin-layer chromatography. Purified microcystin-LR (70, 50, 25 and 10µg ml^{-1}) were spotted on TLC plates and post-chromatographic derivatization was done with Vanillin and Wurster's red reagents. The eluent water-ethyl acetate-propan-2-ol gave better results with the addition of 1ml 5% acetic acid for both derivatizing reagents and a detection limit of 10 $\mu g ml^{-1}$ MC-LR was obtained. However, Wurster's reagent gave more consistent values with a change in retardation factor of 0.06. This protocol was applied to extracts of bloom cultures of *M. aeruginosa* after solid phase extraction on C_{18} cartridges. Two variants of microcystin: MC-LR and MC-LF were visually identified as spots on plates sprayed with Wurster's red reagent. This method can therefore be used for primary detection of microcystins especially in developing country laboratories where instrumental and financial resources are limiting.

KEYWORDS: Microcystins, derivatizing reagents, thin-layer chromatography, visual detection.

INTRODUCTION

The cyanobacteria (blue-green algae) are gram-negative prokaryotes without membrane bound nucleus and organelles. They have only one form of chlorophyll - chlorophyll *a*, and phycobiliproteins which function as accessory pigments in photosynthesis. One class of phycobilins, the phycocyanins, are blue and together with the green chlorophyll *a* are responsible for the blue-green colour of most cyanobacteria.

Under conditions of nutrient enrichment or eutrophication, massive accumulations of cyanobacteria referred to as "blooms" (cell numbers > 10^6 l^{-1}) develop in waterbodies (Carmichael 1992; Nwankwo 1993; Mohamed *et al* 2003). These blooms displace native phytoplankton species contributing to loss of species diversity, clog the gills of fish and invertebrates and on decay cause oxygen depletion in the water.

Certain species of cyanobacteria (*Microcystis, Anabaena, Planktothrix* (*Oscillatoria*), *Aphanizomenon*) also produce toxins which can be bioaccumulated along the food web posing a serious threat to water quality, human and animal health (Unyimadu 2002; Vieira *et al* 2005; Akin-Oriola *et al* 2006). The most frequently encountered group of cyanotoxins are monocyclic heptapeptides – microcystins composed of five amino acids (or derivatives of them) and two variable L-amino acids. There are over 60 variants of microcystins named by the one-letter abbreviation of the L-amino acids e.g. microcystin-LR contains leucine and arginine (Sivonen and Jones 1999).

The toxicity of microcystins is associated with inhibition of protein phosphatases 1 and 2A leading to hepatocyte necrosis, haemorrhage and death. Chronic exposure to microcystins in drinking water promotes tumour development and has been linked to the high incidence of primary liver cancer in some areas of China and Florida (Nishiwaki-Matsushima *et al* 1992, Harada *et al* 1996, Fleming *et al* 2002). In addition, cyanotoxins are not easily degraded in water hence routine monitoring of water bodies is necessary in preventing incidences of cyanotoxin-related poisoning and death.

Traditionally, the toxicity of cyanobacterial cells or blooms is determined by the mouse bioassay which provides a measure of total toxicity within a few hours though it is not very sensitive or specific. Presently, the analytical method of choice is high-performance liquid chromatography - HPLC (Lawton *et al* 1994) however, the initial capital expenditure and technical demands of this method prohibit its applicability in developing countries. Thin-layer chromatography is a cheaper but sensitive alternative and it separates compounds based on their rates of movement on an adsorbent when placed in a suitable solvent. In this study, the adsorbent is a thin layer of silica gel on an aluminium sheet and crude samples which otherwise would clog or damage HPLC columns can be run since TLC plates are used only once. The spots or bands can be visualized by spraying the developed plates with a derivatizing reagent to produce coloured or fluorescent products.

Silica gel TLC has been widely used for the detection of different variants of microcystins (Poon *et al* 1987, Harada *et al* 1988, Ojanpera *et al* 1995) but most methods involve the use of expensive instrumentation like automatic samplers and scanning densitometers. This paper describes non-instrumental visual detection of microcystins by thin-layer chromatography and methods for the extraction and clean-up of toxins from bloom samples.

MATERIALS AND METHODS

Materials

Thin layer chromatography was performed on 5 x 10 cm aluminium-backed silica gel 60 plates with a layer thickness of 0.2 mm (Merck, Germany). The solid phase extraction cartridges were Isolute® C18 (EC) mounted on a VacMaster-10 processing station (International Sorbent Technology, UK).

All reagents were of analytical grade: hydrochloric acid, glacial acetic acid and ethyl acetate were purchased from Fisher Scientific, UK; methanol was from Rathburn, Scotland; sulphuric acid and propan-2-ol were from BDH, UK; potassium permanganate was from Fisons Scientific, England. High-purity water was produced with a Milli-Q system (Millipore, MA, USA).

The derivatizing reagents were 4-hydroxy-3-methoxybenzaldehyde also known as Vanillin (Aldrich Chemicals, Germany) and N,N-dimethyl-1,4-phenylene-diammonium dichloride (N,N-DPDD) also known as Wurster's red reagent (Merck, Germany). Bloom samples were obtained from laboratory cultures of *M. aeruginosa* and microcystin standards (microcystin -LR and -LF) were purified by flash

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chromatography from the cultures (Edwards *et al*, 1996; Lawton and Edwards, 2001).

Preparation of derivatizing reagents

Vanillin

The spraying solution was prepared by dissolving 50 mg of vanillin in a mixture of 8.5 ml methanol, 1 ml glacial acetic acid and 0.5 ml concentrated sulphuric acid. The developed TLC plates were sprayed evenly with this solution and heated at 100 °C for 5 min to aid visualization.

Wurster's red reagent (N,N-DPDD)

For the visualization reaction, developed plates were exposed to chlorine gas for 20 min in a glass tank. The gas was generated by mixing equal volumes of 5 % potassium permanganate and 10 % hydrochloric acid in a filter flask connected to the glass tank. After exposure, the plates were aired in a stream of warm air for 15 s and evenly sprayed with reagent solution. This was prepared by dissolving 100 mg of the reagent in a mixture of 5 ml methanol, 5 ml water and 0.1 ml glacial acetic acid.

Chromatographic conditions

The chromatography chamber was lined with filter paper and filled with eluent (mobile phase) made up of water-ethyl acetate- propan-2-ol (2 + 5 + 3) with the addition of 5 % acetic acid (Pelander *et al* 1997, 2000). The eluent was allowed to saturate the chamber for an hour before plates were developed at ambient temperature.

On each TLC plate, a line was marked in pencil approximately 2 cm from the bottom for applying the sample (i.e. the origin) and another line was marked approximately 1 cm from the top to indicate the eluent front. The volume of sample applied to plates was

10 µl except otherwise stated. Chromatography was performed in ascending mode and plates were placed vertically in the chamber such that the origin was above the surface of the eluent. After development, the retardation factor (R_f) value was calculated as follows:

$$R_f = \frac{\text{distance travelled by substance from the origin}}{\text{distance travelled by the eluent from the origin}}$$

The R_f value is constant for a toxin under a given set of experimental conditions.

Protocol for pure samples

Microcystin-LR was dissolved in methanol to give a range of concentrations - 70, 50, 25, 10 $\mu g ml^{-1}$ for the determination of the detection limit of each derivatizing reagent. Solvent optimisation was carried out using the eluent stated above with the addition of either 0.5 or 1 ml of 5 % acetic acid. The limited quantity of microcystin-LF available did not allow for its use in this assay.

In the second assay, the toxins (microcystin -LR and -LF) were applied to the plate as a mixture in methanol containing $1 \mu g \mu l^{-1}$ of each toxin using Wurster's red reagent while the sample application volume was 20 µl. Pure microcystin-LR and -LF were spotted alongside the mixture to aid identification.

Protocol for bloom samples

Toxins were extracted from bloom samples by suspending 5mg or 50mg of freeze-dried cells of *M. aeruginosa* in 1000 µl of 80 % methanol for an hour. It was then centrifuged at 14,000 rpm for 5 min and 900 µl of the supernatant was diluted with milli-Q water (1:10). The extract was then applied to a C18 (end capped) solid phase extraction cartridge which had been pre-conditioned with 10 ml of methanol and 10 ml of milli-Q water. Solid phase extraction is a rapid clean-up and concentration method for small extracts using short beds of chromatographic packings. After the addition of extract, the cartridge was washed with 10 ml of milli-Q water and 10 ml of 20 % methanol. Toxins were eluted with 80 % methanol (5 ml) and evaporated to dryness under nitrogen in a sample concentrator at 45 °C. The residue was reconstituted with 100 µl of methanol and diluted further (1:2.5) before applying a 10 µl volume to the TLC plate. Microcystin standards (MC-LR and MC-LF) were also spotted alongside the samples.

RESULTS AND DISCUSSION

There are various chromatographic methods for the detection of microcystins but the need for complex instrumentation has increased the financial outlay beyond the resources of small-scale laboratories doing primary monitoring work. TLC with visual detection is a technique that enables full non-instrumental analysis with consequent reduction in costs. In this study, visual detection of microcystins by post-chromatographic derivatization was investigated. Both derivatizing reagents gave coloured products on plates: violet-grey spots on pale pink background for Vanillin and aniline red spots on pink background for Wurster's red reagent.

The addition of 5 % acetic acid enhances miscibility of eluents and clarifies spots on plates. Solvent optimisation of acetic acid with various concentrations of pure microcystin-LR showed that more spots were visible with 1ml of acetic acid than with 0.5 ml for vanillin (Table I). Although with Wurster's reagent, visibility of spots seemed unaffected by the amount of acetic acid. Also the limit of detection of pure microcystin-LR was higher with Wurster's reagent (10 $\mu g ml^{-1}$) irrespective of the amount of acid.

Whilst the lower detection limit (25 $\mu g ml^{-1}$) obtained with Vanillin varied with amount of acid in eluent. Pelander *et al* 2000 reported a detection limit of 1 $\mu g ml^{-1}$ for pure microcystin-LR with Wurster's reagent thus it is possible to use this method in detecting microcystins given the World Health Organisation guide line of 1 ug l^{-1}.

The change in R_f values (ΔR_f) seemed lower with Vanillin (Table 1) but this could be a direct result of the fewer number of spots. Following from the above 1 ml of acetic acid is considered optimal for the detection of pure microcystin-LR and Wurster's red is the preferred derivatizing reagent under the given chromatographic conditions.

Table II lists R_f values for spots obtained from the derivatization of pure and mixed microcystin-LR and -LF using Wurster's reagent. There was no significant change in R_f values when toxins are spotted as a mixture hence the low values obtained.

The applicability of the derivatization reactions in screening for microcystins in bloom samples was tested with laboratory cultures of *M. aeruginosa*. Initially, when extracts were applied to plates without first carrying out solid phase extraction, no spots were observed as the biological matrix interfered with analysis.

A clean-up step was therefore introduced and the results shown in Table III indicate that four bands or spots were observed for each weight of sample. By comparison with the R_f values of standards, two of the spots (R_f 0.66 and 0.83) were identified as microcystin-LR and –LF respectively. However the lack of standards of other microcystins precluded the identification of other bands. The spots obtained from the 50 mg sample had a more intense (deeper) colouration but the R_f value for each microcystin was constant irrespective of weight of algal cells extracted.

Although thin-layer chromatography was used here for qualitative screening of microcystins, since the intensity of spots obtained was concentration-dependent it is possible to modify it for quantitative screening of extracts by spotting various concentrations of the standards along with samples.

CONCLUSION

Visual post-chromatographic detection of algal toxins was carried out. The derivatizing reagent, Wurster's red gave

consistent and clearer bands on plates hence it is preferred for detecting microcystins in bloom samples.

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Table I: R_f values obtained for solvent optimization of acetic acid using pure microcystin-LR. (a) Eluent with 0.5 ml of 5 % acetic acid (b) Eluent with 1ml of 5 % acetic acid.

a)

	Derivatizing Reagent	
Concentration of MC-LR ($\mu g\ ml^{-1}$)	Vanillin	Wurster's red
70	0.66	0.63
50	0.65	0.63
25	ND	0.77
10	ND	0.68
	ΔR_f = 0.01	ΔR_f = 0.14

b)

	Derivatizing reagent	
Concentration of MC-LR ($\mu g\ ml^{-1}$)	Vanillin	Wurster's red
70	0.74	0.75
50	0.73	0.79
25	0.77	0.74
10	ND	0.73
	ΔR_f = 0.04	ΔR_f = 0.06

ND = No visible spot detected. Data presented are a representative set from repeated assays.

Table II: Detection of pure and mixed microcystin-LR and -LF using eluent with 5 % acetic acid and wurster's red reagent.

Toxin	R_f values	ΔR_f
MC-LR (alone)	0.56	
MC-LR (mixture)	0.56	0.00
MC-LF (alone)	0.80	
MC-LF (mixture)	0.81	0.01

Data presented are a representative set from repeated assays. (Toxin concentration was $1\mu g\ \mu l^{-1}$ while application volume was 10 µl for each toxin).

Table III: R_f values for standards (microcystin-LR and -LF) and cultured bloom samples of *M. aeruginosa* using 5 % acetic acid (1ml) and wurster's red reagent.

Standards	Extract from 50 mg of freeze-dried algal cells	Extract from 5 mg of freeze-dried algal cells
MC-LR 0.64	1st band 0.66	1st band 0.66
MC-LF 0.81	2nd band 0.75	2nd band 0.74
	3rd band 0.83	3rd band 0.83
	4th band 0.89	4th band 0.88

Data presented are a representative set from repeated assays.

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