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 10 μg 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 μg 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 C₁₈ 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.
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.

Wurstler'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 (Rf) 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 Rf 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 µg/ml 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 mg/ml of each toxin using Wurstler's red reagent while the valid 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 5 mg or 50 mg 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 concentrafr 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 by 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 Wurstler'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 1 ml of acetic acid than with 0.5 ml for vanillin (Table 1). Although with Wurstler'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 Wurstler's reagent (10 µg/ml) irrespective of the amount of acid.

Whilst the lower detection limit (25 µg/ml) obtained with Vanillin varied with amount of acid in eluent (Pelander et al., 2000) reported a detection limit of 1 µg/ml for pure microcystin-LR with Wurstler's reagent, thus it is possible to use this methodology in detecting microcystins given the World Health Organisation's guideline of 1 µg/l.

The change in Rf values (AR) 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 Wurstler's red is the preferred derivatization reagent under the given chromatographic conditions.

Table II lists Rf values for spots obtained from the derivatization of pure and mixed microcystin-LR and -LF using Wurstler's reagent. There was no significant change in Rf 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 of spots were observed for each weight of sample. By comparison with the Rf values of standards, two of the spots (Rf 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 Rf 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, Wurstler's red gave...
consistent and clearer bands on plates hence it is preferred for detecting microcystins in bloom samples.

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Table 1: Rf 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.

<table>
<thead>
<tr>
<th>Concentration of MC-LR (µg ml⁻¹)</th>
<th>Vanillin</th>
<th>Wurster’s red</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>0.66</td>
<td>0.63</td>
</tr>
<tr>
<td>50</td>
<td>0.65</td>
<td>0.63</td>
</tr>
<tr>
<td>25</td>
<td>ND</td>
<td>0.77</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>0.68</td>
</tr>
</tbody>
</table>

ΔRf = 0.01  ΔRf = 0.14

ND = No visible spot detected. Data presented are 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.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Rf values</th>
<th>ΔRf</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-LR (alone)</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>MC-LR (mixture)</td>
<td>0.56</td>
<td>0.00</td>
</tr>
<tr>
<td>MC-LF (alone)</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>MC-LF (mixture)</td>
<td>0.81</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data presented are a representative set from repeated assays. (Toxin concentration was 1µg µl⁻¹ while application volume was 10 µl for each toxin).

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

<table>
<thead>
<tr>
<th>Standards</th>
<th>Extract from 50 mg of freeze-dried algal cells</th>
<th>Extract from 5 mg of freeze-dried algal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-LR 0.64</td>
<td>1st band 0.66</td>
<td>1st band 0.66</td>
</tr>
<tr>
<td>MC-LF 0.81</td>
<td>2nd band 0.75</td>
<td>2nd band 0.74</td>
</tr>
<tr>
<td></td>
<td>3rd band 0.83</td>
<td>3rd band 0.83</td>
</tr>
<tr>
<td></td>
<td>4th band 0.80</td>
<td>4th band 0.88</td>
</tr>
</tbody>
</table>

Data presented are a representative set from repeated assays.

REFERENCES


