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# Separation of toxic peptides (microcystins) in capillary electrophoresis, with the aid of organic mobile phase modifiers

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**Abstract**

A capillary electrophoretic (CE) method incorporating sodium dodecyl sulphate (SDS)–organic modifier solvents in the CE buffer was developed for the detection of toxic cyclic heptapeptide toxins (microcystins) produced by blue-green algae (cyanobacteria). The applicability of these run buffers for the analysis of microcystins was evaluated and optimum conditions for separation were determined. The migration times, elution order and selectivity of the toxic peptides were influenced by modifying the composition of the electrophoretic buffer with organic solvents [0 to 20% (v/v)]. At maximum addition, the organic solvents with the exception of acetonitrile, increased the viscosity of the buffer solution. In contrast to the migration time of the other microcystins, that of microcystin-RR was not increased by the addition of 2-propanol to the buffer solution. Rather, microcystin-RR eluted more quickly with the increase in 2-propanol, thereby effecting changes in the elution order of the microcystins. In addition, this solvent resulted in comigration of microcystin-LR and microcystin-YR. No significant relationship was found between the elution order and separation and the structure of the toxic peptides studied in micellar electrokinetic capillary chromatography with an organic modifier in the buffer solution; but there is an agreement between the effects of the organic modifiers and their dipole moments. Parameters such as linearity, sensitivity and reproducibility were also evaluated. High-efficiency separations of toxic peptide molecules having equal or nearly equal mass to charge ratios have been achieved using SDS as an additive to the running buffer. The influence of the pH has been examined.

*Keywords:* Microcystins; Peptides

## 1. Introduction

Capillary electrophoresis (CE) is becoming an increasingly important tool for the analysis of a wide range of molecules. The speed of CE analysis, its high efficiency and versatility have promoted the development of new applications using this method [1–4].

Micellar electrokinetic capillary chromatography (MECC) [5,6], is a high-resolution separation technique for separating small neutral molecules as well as charged solutes. Many types of molecules are amenable to MECC, including amino acids [7], polypeptides [8], chiral substances [9] and pharmaceuticals [10]. Liu et al. [11] have reported the MECC separation of small peptides having similar charges while Yashima et al. [12] have successfully separated closely related large peptides with the MECC technique. However, the use of MECC for the separation of cyanobacterial toxins has not previously been investigated; therefore we are studying the effects of organic modifiers on the separation

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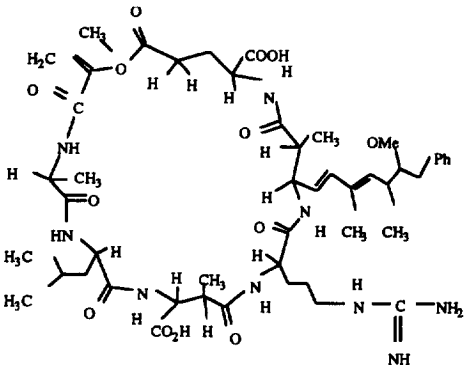
of microcystins produced mainly by the blue-green alga (cyanobacterium) *Microcystis aeruginosa* [13]. These toxins have been implicated in human health problems [14–16]. Cyanobacterial blooms are common in eutrophic freshwaters and brackish waters. The structures of the four toxic peptides [16,17] studied are shown in Fig. 1.

Due to the adverse biological and human health effects of microcystins, there is a need for rapid analytical techniques that can provide qualitative and quantitative detection of the toxins. Although mouse bioassay is widely used to detect microcystins [18,19], it lacks quantitation and sensitivity. In order to discriminate between the different microcystins, analytical techniques that offer a high resolution are required. Reversed-phase high-performance liquid chromatography (RP-HPLC), which separates compounds based on their hydrophobic character, represents one of the most powerful techniques currently available for the analysis, characterisation and quantitation of microcystins [16,20]. While the resolution of microcystin-LR (M-LR), microcystin-YR (M-YR) and microcystin-RR (M-RR) is quite good, this technique can involve relatively lengthy analysis time as well as high solvent and chemical consumption.

MECC, a recently developed form of capillary zone electrophoresis technique, employs a surfactant above the critical micelle concentration and permits the separation of neutral and uncharged molecules in an electroosmotically driven system [7,8]. Migration times of analytes in MECC systems are affected by the electrophoretic mobility of the analyte, the interactions between the analyte molecules and the



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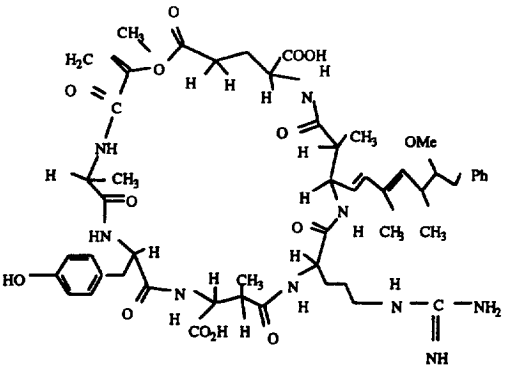

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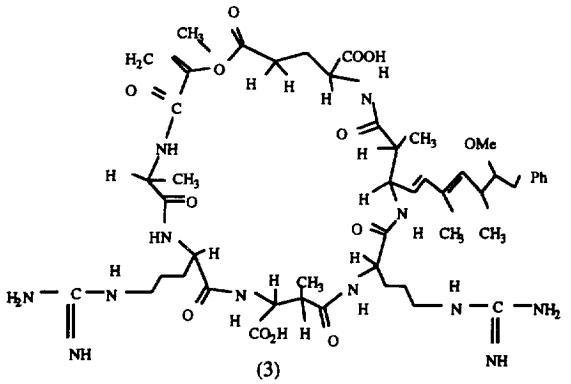

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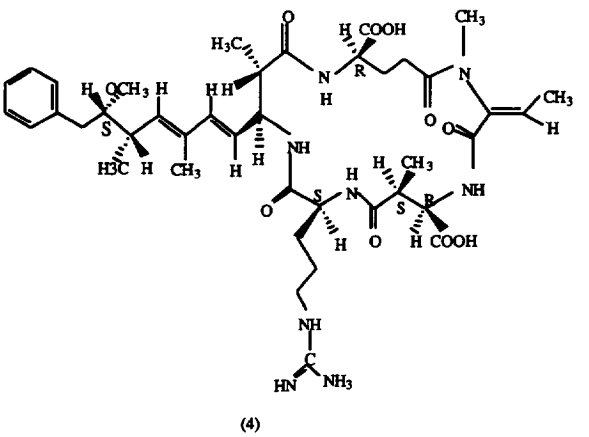

(4)

Fig. 1. Structures of the toxic peptides: (1) microcystin-LR, (2) microcystin-YR, (3) microcystin-RR and (4) nodularin.

micelles and the magnitude of electroosmotic flow [10].

In this study, we focused on the application of MECC with organic modifiers for the separation of microcystins using UV detection, which is the most popular detection method in CE. Sodium dodecyl sulphate (SDS), a common surfactant for MECC was used. Any evaluation of CE as a viable alternative for solving a given analytical task (e.g. in peptide analysis) should be based on resolution, selectivity, peak capacity, duration and cost of the analysis.

In accordance with these needs, our aim was to develop a CE method (utilizing buffers modified with organic solvents) suitable for the analysis of microcystins. In this work, the effects of buffer pH, ionic strength and SDS concentration were systematically evaluated for their effects on the elution, resolution and separation of three microcystins (M-LR, M-YR and M-RR) and nodularin. We also evaluated three organic modifiers (acetonitrile, methanol and 2-propanol) of different selectivity for potential use in MECC buffer solutions. This method has been optimized for highly efficient separation of some toxic cyclic heptapeptides having equal or nearly equal mass to charge ratios.

## 2. Experimental

### 2.1. Instruments

All work was performed on a Beckman P/ACE 2050 CE system, using a 50 $\mu$m I.D, 50 cm long fused-silica capillary, an operating voltage of 20 kV and a UV detector set at 230 nm. Data were collected and processed from the instrument using System Gold software (Beckman instruments).

### 2.2. Materials

The reagents (test solutes) used in the analysis were M-LR, M-YR and M-RR. All microcystins were purchased from Calbiochem. Nodularin, a pentapeptide with similar biogenesis and bio-activity to microcystin, (from Novabiochem, Sydney) and Pheu-leu-glu-glu-leu (PLGGL) were used as internal standards. The SDS was obtained from Aldrich. HPLC-grade acetonitrile (May and Baker), methanol, and 2-propanol were purchased from Ajax Chemicals. Other reagents used were of analytical grade. Distilled deionized water was used.

### 2.3. Procedure

The fused-silica column was treated with 0.5 *M* NaOH for 10 min and subsequently with Milli-Q water (Millipore) for 5 min before introducing the electrophoresis buffer for 5 min. When changing buffer composition, the capillary was rinsed for 5 min with 0.5 *M* NaOH and subsequently with Milli-Q water for 3 min before introducing the new buffer. A stock buffer solution of 0.1 *M* sodium tetraborate adjusted to pH 8.03, 8.32, 8.47 and 8.85 with 0.4 *M* boric acid was used. The buffer system was composed of SDS, buffer stock solution and Milli-Q water. All the micellar buffer solutions were filtered through 0.45-$\mu$m membrane filters (Millipore).

Microcystin stock samples in concentration of 333.0 $\mu$g/ml in 20% methanol were used in the CE analysis. A mixture consisting of 1.0 $\mu$g M-LR, 1.0 $\mu$g M-YR, 1.0 $\mu$g M-RR, was used to study the separation mechanisms involved and will subsequently be referred to as the test mixture. Nodularin in 20% methanol was used as an internal standard for the migration of the microcystins in the micelles. Four injections were made for each peptide sample at every buffer composition. Sample injection was accomplished by pressure for 5 s. The data sample rate was 8/s.

## 3. Results and discussion

### 3.1. Effect of pH

In preliminary experiments, an arbitrary pH of 8.5 and SDS concentration of 30 m*M* were used, but as a more thorough understanding of parameter interdependency was desired, these conditions were used as a starting point for further method development. The internal standard PLGGL was chosen arbitrarily due to its availability. The effect of pH on the separation of the microcystins in the test mixture was investigated over the pH range 8.0 to 9.0. The best separation between M-LR and M-YR occurred at pH

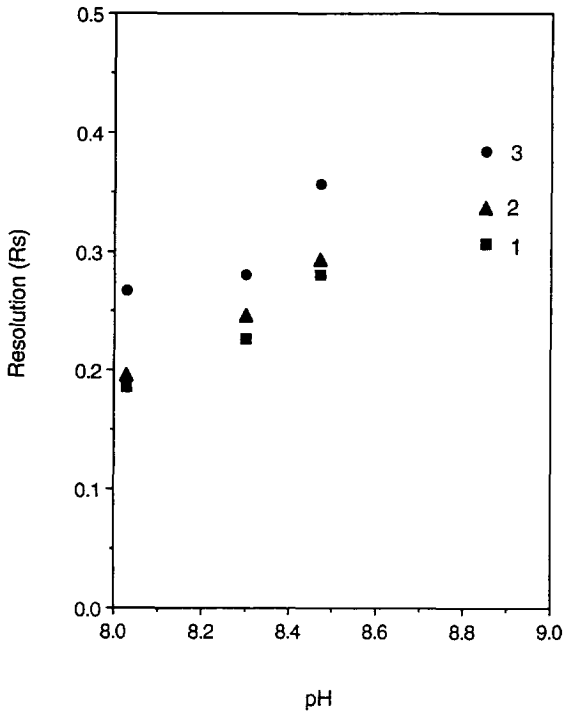


Fig. 2. Effect of pH on resolution ($R_S$) of M-YR (1); M-LR (2) and M-RR (3). A buffer of 0.4 *M* boric acid and 0.1 *M* sodium tetraborate at the specified pH and a 50 $\mu$m×50 cm capillary operated at 20 kV was used. The samples were 5.0 $\mu$g/ml in 20% MeOH, with a 5-s injection time.

in excess of 8.8 (Fig. 2) and due to the proximity of the borate p$K_a$ of 9.14, a pH of 8.85 was chosen for subsequent method development. The variation of pH did not cause any change in elution order. However, the migration times of the peptides decreased with increasing pH. Similar behaviour (decrease in migration times with increase in pH) was observed in the separation of closely related large peptides [12].

## 4. Effect of SDS concentration on the separation of peptides

Several ionic surfactants have been shown to improve selectivity of separation in CE when added to the running buffer [1–10]. However, in order to separate closely related species, high concentrations of these surfactants are necessary. SDS has been investigated in MECC [21] where it was shown that SDS was the most important factor for analyte velocity and with optimized SDS and borate concentration, good separation and baseline resolution could be obtained.

Resolution was still inadequate at the arbitrary settings, hence the buffer ionic strength was varied to achieve further optimisation. At an applied voltage of 20 kV and 30 m*M* SDS, the borate concentration of the buffer was increased from 10 to 50 m*M* with minimal significant joule heating. The progressive increase in the buffer ionic strength from 0 to 25 m*M* of borate improved the peak resolution of the three microcystins (Fig. 3).

Five concentrations of SDS (0–90 m*M*) were trialled in a running buffer of 17.5 m*M* borate. The addition of SDS progressively changed the migration times of the peptides (Fig. 4). This result suggested that differential partitioning of the analytes into the mobile micelles had occurred [22,23]. MECC can separate both neutral and ionic substances by partitioning the analyte between two phases which have different migration times, that is a carrier (SDS) and the surrounding medium [5]. The addition of 60 m*M* SDS gave the best baseline separation of the three peptides (Fig. 4), hence this was taken as the optimum SDS concentration.

The addition of SDS retarded migration and did not affect selectivity. However, the internal standard (PLGGL) eluted at the solvent front, thereby making integration and accurate quantitation of analytes impossible. Therefore, further optimization was necessary to select an internal standard which eluted with good resolution and distinct from the solvent peak. This was achieved by using nodularin as the internal standard and introducing organic modifiers to the buffer. The electropherograms of the test peptide mixture plus nodularin, with and without SDS added to the running buffer are compared in Fig. 5. The migration-time window [the interval between the migration time of the bulk solution ($t_o$) and that of the micelle ($t_{mc}$)] was three times wider using SDS than without SDS. This was further widened with good resolution by the introduction of acetonitrile or methanol to the running buffer as explained in the next section.

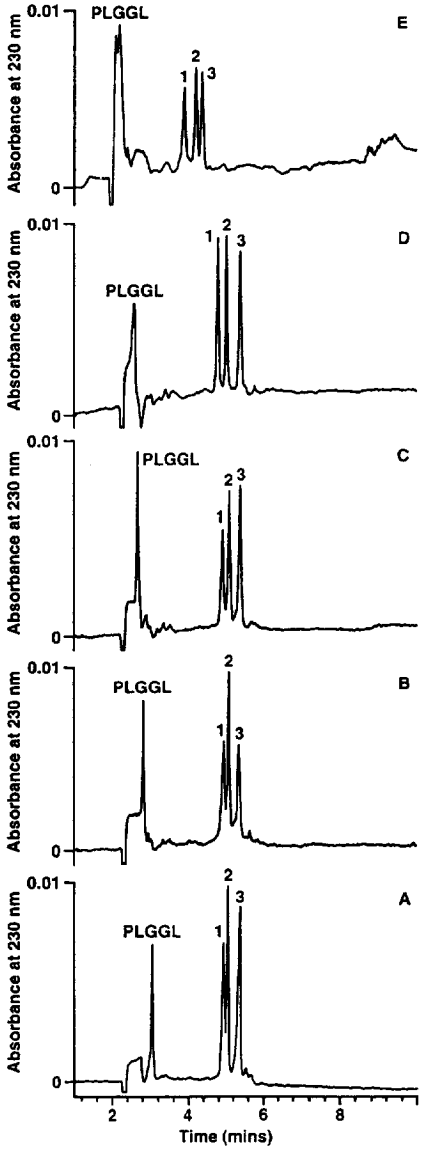


Fig. 3. Effect of borate concentration on migration time and peak resolution. (A) 10 m*M*; (B) 15 m*M*; (C) 17.5 m*M*; (D) 25 m*M* and (E) 50 m*M*. Abbreviation used: PLGGL=Pheu-leu-glu-glu-leu. (1) M-LR; (2) M-YR and (3) M-RR. All other conditions are as presented in Fig. 2.

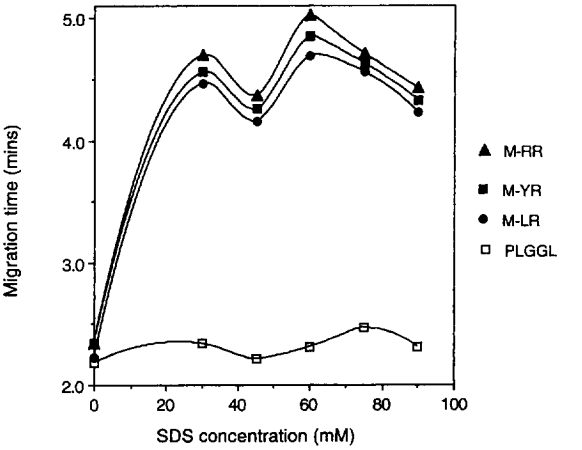


Fig. 4. Effects of SDS concentration on migration time. A buffer of 0.4 *M* boric acid and 0.1 *M* sodium tetraborate pH 8.85, 17.5 m*M* SDS and a 50 $\mu$m×50 cm capillary operated at 20 kV was used. The samples were 1.0 $\mu$g/ml in 20% MeOH, with a 5 s injection time. Other conditions as outlined in the text.

## 5. SDS–Organic modifier system

The peak resolution in MECC can be improved by modifying the buffer with organic solvents which decrease the electroosmotic flow and the affinity of hydrophobic solutes for the micellar phase [6,24]. Alcohols and acetonitrile were tested as organic modifiers with differing results. The alcohols increased the migration time of each analyte while the reverse was the case with acetonitrile. The degree of increase of the migration time caused by the alcohol at a given concentration agreed with other observations [24]: the longer the carbon chain of the alcohol, the greater the increase in migration time. The migration window was increased by 30% with the addition of 5% acetonitrile and 28% with 10.0% methanol.

The addition of 2-propanol affected selectivity, M-RR eluted before M-LR and M-YR which comigrated. The migration of M-LR is illustrated by the increased migration time ($t_m$) and the crossed line in Fig. 6 which shows the migration times of M-LR as a function of the percentage concentration of the different organic modifiers.

The electropherograms of the microcystin test mixture using the optimal separation conditions (Fig. 7) reveals that resolution was >1. A resolution value <1 is considered poor, therefore this buffer mixture not only gives excellent separations, but also has the potential for use in routine analysis.

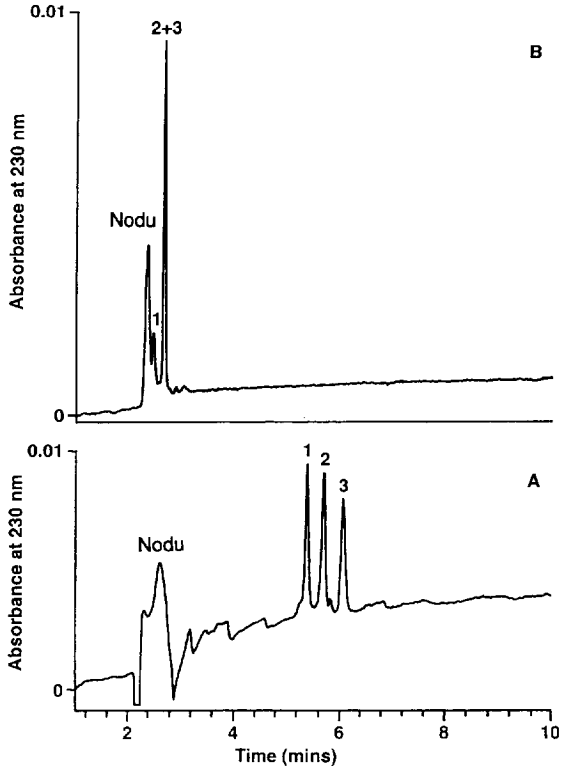


Fig. 5. Electropherograms of 3 test substances with (A) 60 m*M* of SDS and (B) no SDS added to the running buffer. Abbreviation used: Nodu=nodularin. (1) M-LR; (2) M-YR and (3) M-RR. The samples were 1.0 $\mu$g/ml in 20% MeOH, with a 5-s injection time. All other conditions are as presented in Fig. 2.

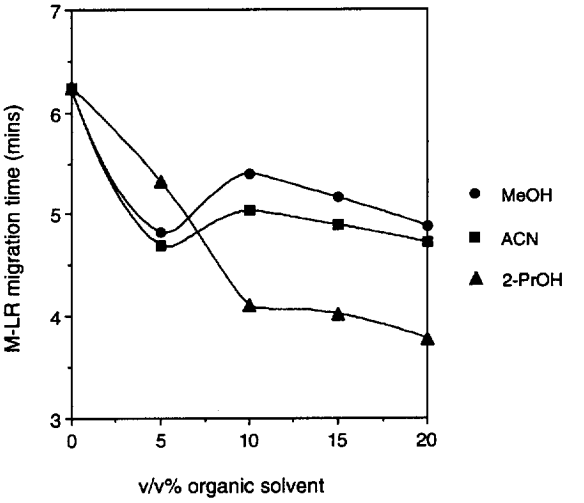


Fig. 6. CE migration times of microcystin-LR as a function of the percentage concentration of the different organic modifiers. (1) ACN; (2) MeOH and (3) 2-PrOH. pH 8.85, 25 m*M* SDS. Other conditions as outlined in the text.

## 6. Linearity and reproducibility

In order for an analytical technique to perform quantitatively, it needs to be both linear and reproducible. Linearity over a wide dynamic range eliminates the need for multiple level calibration curves. The linearity range for the various microcystins was determined by plotting the concentration of each analyte against the ratio of the peak area of the analyte to the peak area of the internal standard (nodularin) maintained at constant concentration. The calibration curve generated by serial dilution from 100 mg/l to 0.3 mg/l M-LR was linear up to 66 mg/l.

The reproducibility was evaluated by analysing seven replicates of 5 $\mu$g/ml of M-LR solution as a representative sample. The relative standard deviation was 1.41% and the correlation coefficient was 0.9983. The detection limit (defined as three times the baseline noise) is 0.12 $\mu$g/l. On the other hand, the limit of quantification defined as 10 times the baseline noise is 0.85 $\mu$g/l.

## 7. Conclusion

The results demonstrate that CE is a fast and easily applicable method in the separation of toxic peptides with similar charge-to-mass ratio. The four peptides investigated here can be separated within 5 minutes. We can influence the migration time, elution order, baseline separation and selectivity of these peptides using the CE system, by modifying the composition of the buffer with organic solvents. We note that various parameters such as charge, temperature, concentration of surfactant and voltage can also affect the separation of these peptides. Nevertheless, the sensitivity, resolution, speed and cost of CE analysis makes it a superior analytical method.

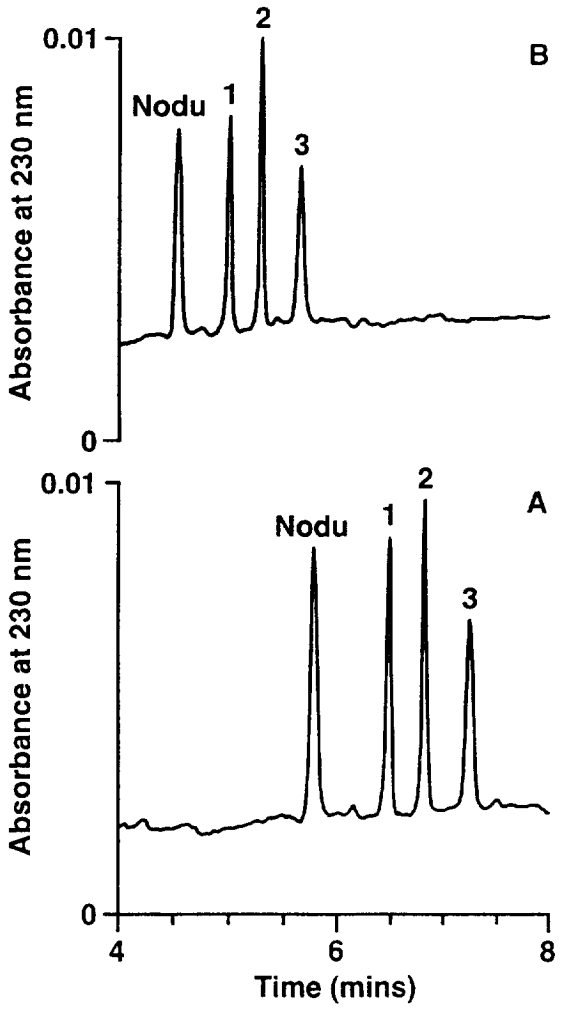


Fig. 7. Electropherograms of the optimum separation of the 3 microcystins and nodularin by MECC using (A) 5% ACN–SDS system and (B) 10% MeOH–SDS system; 25 m*M* borate, 60 m*M* SDS and pH 8.85. Abbreviation used: Nodu=nodularin.

## Acknowledgments

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