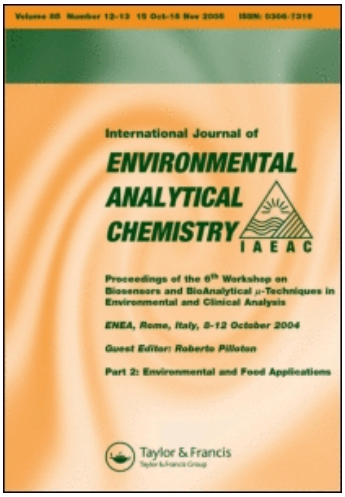


International Journal of Environmental Analytical Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

An Application of Capillary Electrophoresis for the Analysis of Algal Toxins from the Aquatic Environment

Ana Gago-Martínez[a]; José Manuel Leão[a]; Nuria Piñeiro[a]; Esther Carballal[a]; Estela Vaquero[a]; María Nogueiras[a]; José A. Rodríguez-Vázquez[a]

[a] Departamento de Química Analítica y Alimentaria, Universidad de Vigo, Vigo, Spain

Online publication date: 17 September 2010

To cite this Article Gago-Martínez, Ana , Manuel Leão, José , Piñeiro, Nuria , Carballal, Esther , Vaquero, Estela , Nogueiras, María and Rodríguez-Vázquez, José A.(2003) 'An Application of Capillary Electrophoresis for the Analysis of Algal Toxins from the Aquatic Environment', International Journal of Environmental Analytical Chemistry, 83: 6, 443 — 456

To link to this Article: DOI: 10.1080/0030673103100011706

URL: http://dx.doi.org/10.1080/0030673103100011706

Intern. J. Environ. Anal. Chem.
Vol. 83, No. 6, pp. 443–456, June 2003

Taylor & Francis
Taylor & Francis Group

AN APPLICATION OF CAPILLARY ELECTROPHORESIS FOR THE ANALYSIS OF ALGAL TOXINS FROM THE AQUATIC ENVIRONMENT

ANA GAGO-MARTÍNEZ*, JOSÉ MANUEL LEÃO, NURIA PIÑEIRO, ESTHER CARBALLAL, ESTELA VAQUERO, MARÍA NOGUEIRAS and JOSÉ A. RODRÍGUEZ-VÁZQUEZ

Departamento de Química Analítica y Alimentaria, Universidad de Vigo, Campus de Vigo, 36200 Vigo, Spain

(Received 4 October 2002; in final form 24 February 2003)

In this work capillary electrophoresis (CE) with UV detection has been applied to the analysis of different natural toxins produced in the aquatic environment. This technique is presented as an alternative to other chemical techniques such as HPLC, and the optimisation of analytical methodologies was carried out for diverse marine toxins including Paralytic and Amnesic and some polyether toxins, such as Yessotoxins, as well as for certain microcystin toxins produced by cyanobacteria present in freshwaters.

Sample preparation steps were optimised and adequate electrophoretic conditions developed for achieving a complete separation of compounds with similar structures involved in such contamination. The influence of the biological matrices where they are involved has also been studied and the potential use of CE-UV as a tool for monitoring these aquatic toxins is also discussed.

Keywords: Capillary electrophoresis; PSP toxins; ASP toxins; Yessotoxins; Microcystins; Aquatic toxins

INTRODUCTION

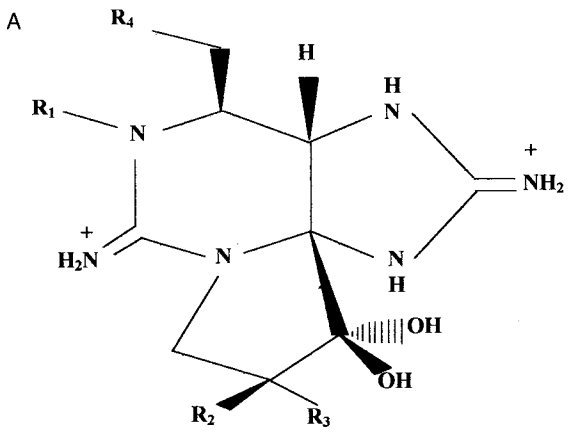
Contamination of marine and freshwater environments by algal toxins is an important issue because of the global socioeconomic impact of these toxins on human health and fisheries industries. Many algal blooms appear to be stimulated by eutrophication brought on by domestic, industrial and agricultural wastes. Algal toxins have been of particular concern in recent years due to this growing problem and their high potency. Many scientists in the field have therefore been paying attention to these compounds, trying to find and develop sensitive analytical methodologies for their control.

Paralytic, Diarrhetic and Amnesic Shellfish Poisoning (PSP, DSP and ASP) toxins are the main groups of toxins found in the marine environment, which have been produced by phytoplanktonic species [1,2]. Yessotoxins (YTX) had been included in

*Corresponding author. Fax: +34-986-812556. E-mail: anagago@uvigo.es

ISSN 0306-7319 print: ISSN 1029-0397 online © 2003 Taylor & Francis Ltd
DOI: 10.108/0030673103100011706

the DSP group, although nowadays owing to their non-diarrhetic symptomatology, which is mainly cardiotoxic [3], these compounds are considered as a separate group of marine toxins, having been found in several parts of the world and seeming to be an increasing problem in these areas. Microcystins are hepatotoxins produced by genera of cyanobacteria (blue-green algae) from freshwaters; these compounds have been responsible for animal and human intoxications reported in several regions [4]. Figure 1 shows the structures of the toxins mentioned above.



			Carbamate Toxins	N-Sulfocarbamate Toxins	Decarbamate Toxins
R1	**R2**	**R3**	**R4= -OONH$_2$**	**-OONHSO$_3^-$**	**-OH**
H	H	H	STX	GTX5	dcSTX
H	H	OSO3-	GTX2	C1	dcGTX2
H	OSO3-	H	GTX3	C2	dcGTX3
OH	H	H	NEO	GTX6	dcNEO
OH	H	OSO3-	GTX1	C3	dcGTX1
OH	OSO3-	H	GTX4	C4	dcGTX4

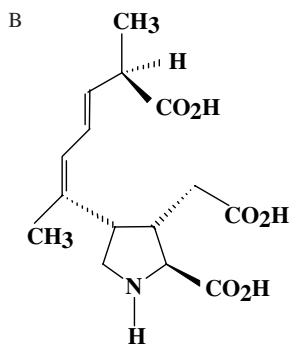


FIGURE 1 Chemical structure of different aquatic toxins: A PSP toxins; B domoic acid; C yessotoxin; D microcystins.

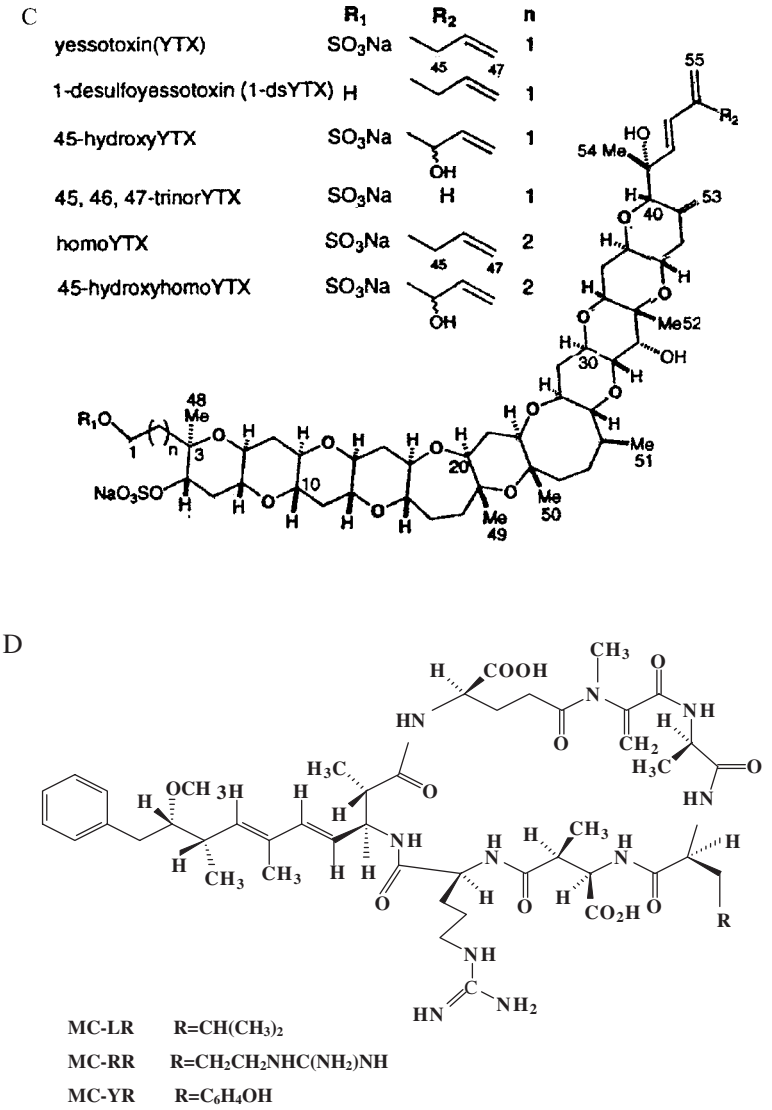


FIGURE 1 Continued.

A number of analytical methodologies have been proposed for the analysis of the compounds described, such as immunochemical, enzymatic or radioimmunoassays [5,6], liquid chromatographic methods being the most widely used with both UV and fluorescence detection [7–10]. Capillary electrophoresis (CE) has been recently applied as an alternative for the analysis of algal toxins [11,12]; the separation in this case is based on the different mobilities of these compounds in an electric field depending on the charge and the size of the molecules. This technique is capable of high efficiency and resolution. The main advantages of using CE in comparison with HPLC include simplicity, rapid method development, fast analysis times, low cost and a variety of separation modes. In addition, CE also requires minimal quantities of reagents and

separation media, thus avoiding the large volumes of organic waste associated with HPLC methods.

High-Performance Capillary Electrophoresis has been applied in this study for the analysis of the algal toxins mentioned above. Several electrophoretic parameters have been optimised for further application to the analysis of samples naturally contaminated with these toxins. Sample preparation has also been an important step to consider owing to the complexity of the matrix where these compounds are present. Different SPE procedures have been applied to the extraction and concentration of the named compounds with the aim of removing interferences as well as contributing to an increase in the sensitivity specially required for CE.

EXPERIMENTAL

Toxin Standards and Samples

Domoic acid calibration solution (DACS-1B) and Mussel Tissue Reference Material (MUS-1) containing 100 μg DA/mL and 100 μg DA/g respectively were provided by the Marine Analytical Chemistry Standards Program, National Research Council of Canada. Standards of PSP toxins in 0.03 M acetic acid, STX and dcSTX, provided by RIVM (Bilthoven, The Netherlands) for BCR Standard Measurements and Testing Program Certification Study, were used for this study. YTX standard was kindly provided by Professor Yasumoto (Japan Food Research Laboratory, Tokyo, Japan) and standard solutions of Microcystins LR, YR and RR (MC-LR, MC-YR and MC-RR) were purchased from Calbiochem-Novabiochem (Nottingham, UK).

Razor clams and mussels contaminated with ASP from Ría de Vivero (Lugo), were kindly provided by Delegación Provincial de Pesca de Lugo, Consellería de Pesca, Xunta de Galicia. Mussel samples contaminated with PSP from Ría de Vigo were provided by Consellería de Sanidade, Xunta de Galicia and samples contaminated with YTX consisted of green-shell mussels from New Zealand. For Cyanobacterial samples, contaminated algae from a natural bloom in Montargil (Portugal) were provided by Dr Susana Franca from Instituto Nacional da Saúde Dr. Ricardo Jorge, Lisboa, Portugal. All these samples were kept frozen (−18°C) until analysis.

Reagents

Methanol, acetonitrile and other solvents were analytical grade. Distilled and deionised water (Milli-Q Water Systems, Millipore, Bedford, MA, USA) was used in the preparation of all aqueous solutions.

Guaranteed grades of reagents such as boric acid, morpholine, formic acid, sodium dodecyl sulphate and sodium borate were used in the different analyses.

CE System

Analyses were performed in a HP ^{3D}CE system (Hewlett-Packard, Agilent Technologies) equipped with a diode array detection system (DAD) and HP ^{3D}Chemstation data analysis software.

Analysis of PSP Toxins

Extraction and Clean-up

The official method of the Association of Official Analytical Chemists (AOAC) [13] was used for extraction of PSP toxins in mussel samples with some slight modifications. A portion of 3 mL of extract was passed through a C_{18} cartridge (3-mL cartridge supplied from Waters) for SPE previously conditioned with methanol and water under the conditions described by Leão *et al.* [14] and the portion of eluent between 1.5 and 2 mL was collected for the analysis.

All extracts were ultrafiltered through a 0.45-μm membrane (Ultrafree-MC, Millipore) prior to injection into the CE system.

CE-UV Analysis of PSP Toxins

CE analysis of these compounds was carried out under similar conditions to those reported by Locke and Thibault with some slight modifications [15]. A polyvinylalcohol (PVA) capillary with 104 cm as total length and 0.75-μm i.d. with extended light path provided by Hewlett-Packard was used for the separation of PSP components. The settled wavelength was 200 nm and 20 kV the constant applied voltage. Hydrodynamic injection was used for introduction of the sample into the capillary and a pressure of 50 mbar was applied for the time needed to fill 20% of the volume of the capillary. Isotachophoresis (CITP) was used as a preconcentration mode and was performed using 50 mM morpholine in water adjusted to pH 5 with formic acid as the background and leading electrolyte and 10 mM formic acid as terminating electrolyte.

Analysis of ASP Toxins

Extraction and Clean-up

To 4 g of homogenated shellfish tissue, 16 mL of methanol–water (1 : 1 v/v) was added. After homogenisation of the mixture for 3 min and centrifugation at 4500 r.p.m for 10 min, the supernatant was then filtered through a 0.45-μm filter (Millex-HV) and 5 mL of this extract were taken for clean-up purposes. This clean-up was carried out in two steps using both anion- and cation-exchange SPE cartridges, following the conditions proposed by Zhao *et al.* [11] with slight modifications [16].

In the first step 5.0 mL of extract was passed through a strong anion-exchange cartridge (SAX, Part. No.1210-2044, lot. No.182639, 3 mL, 500 mg, Varian) previously conditioned with 6 mL methanol, 3 mL water and 3 mL methanol–water (1 : 1 v/v). The extract was then washed with 3 mL methanol : water (1 : 1) and the elution was carried out using 5 mL of 0.1 M formic acid. The eluate was then passed through a cation-exchange (SCX, Part. No. 1211-3039, lot No. 171069, MFG Code 0290, 10 mL, 500 mg, Varian) preconditioned with 3 mL of methanol, water and 0.1 M formic acid. 5 mL of SAX eluate was loaded before and then washed with 5 mL 0.01 M formic acid, and the toxins were then eluted with 0.5 mL of 25 mM sodium tetraborate (pH = 9.2)–acetonitrile (9 : 1 v/v). Six portions of 2 mL of this last eluate were taken and the ASP toxins started to appear in the third portion.

CE-UV Analysis of ASP Toxins

CE analysis of ASP toxins were carried out using a 66 cm × 363 μm O.D., 50-μm i.d. bare fused-silica capillary with a UV window located 15 cm from the end of the capillary at room temperature. The UV detection was performed at a wavelength of 242 nm. Hydrodynamic injection was done using a 50 mbar push for 12 s and the voltage applied was 30 kV. Different buffer electrolyte concentrations in the range 10, 25 and 50 mM in borate buffer were used.

Analysis of Yessotoxin

CE analysis of YTX was carried out following the procedure suggested by Prof. Yasumoto (personal communication) with some slight modifications. Detailed information about this CE procedure will be given in Gago-Martínez *et al.* (manuscript in preparation).

Extraction and Clean-up

Methanol–water (8 : 1 v/v) was the solvent used for extraction of YTX; typically 9 mL of the solvent was used for 3 g of shellfish tissue. The supernatant obtained after homogenisation and centrifugation was then evaporated to dryness and redissolved in 20 mM phosphate at pH 5.8.

SPE was carried out in Sep-Pak Plus C_{18} cartridges (Waters, Mildford, MA, USA) previously conditioned with 20 mM phosphate at pH 5.8. After loading 20 mL extract, 6 mL methanol–water (2 : 8 v/v) solution was used for the washing and finally the toxin was eluted with 6 mL methanol–water (7 : 3 v/v). After evaporation to dryness and redissolution of the eluate into 200 μL of methanol, CE/UV analysis was performed under the conditions previously described.

CE-UV Analysis of YTX

The capillary used for this analysis was a bare fused-silica capillary, effective length 56 cm, previously conditioned with background buffer. The wavelength was settled at 230 nm and a buffer electrolyte of 100 mM phosphate at pH 8.5 was used with 20% methanol as an organic modifier.

Hydrodynamic injection at 50 mbar for 20 s was used for sample introduction in the capillary, while the separation was carried out at 20 kV during 30 min.

Analysis of Microcystins

Extraction and Clean-up

Lyophilised cyanobacterial cells were extracted and cleaned up following the procedures described by Bateman *et al.* [17] with some slight modifications. A 0.5 g aliquot of the toxic material was extracted with 50 mL of 25% MeOH. After sonication for 10 min, stirring for 20 min and centrifugation at 4000 r.p.m., the supernatant was collected and the pellets re-extracted twice using the same procedure. Combined supernatants were concentrated in a rotary evaporator to a final volume of 15 mL.

Bakerbond Octadecyl with 500 mg ODS columns (J.T. Baker, Phillipsburg, NJ, USA) were used for SPE of the algal extracts. After conditioning with 5 mL of acidified methanol (MeOH–0.1%TFA), 5 mL of 100% methanol and water, 0.5 mL of the extract was loaded; the cartridge was then washed with 2×10 mL of water and microcystins eluted using 10 mL of methanol–0.1%TFA. The eluate was evaporated and the residue redissolved in 100 μL of methanol for CE analysis.

CE-UV Analysis of Microcystins

CE in the micellar electrokinetic chromatography (MEKC) mode was performed in bare fused-silica capillaries, 55-cm effective length × 50-μm i.d. The background electrolyte, consisting of 20 mM sodium dodecyl sulphate (SDS) as anionic surfactant in 120 mM borate buffer, was adjusted to pH 9.5 with sodium hydroxide. The separation voltage was 25 kV applied in the capillary after introducing the sample by hydrodynamic injection with a pressure of 50 mbar during 5 s. A solution of 30-mM borate at pH 9.5 (adjusted with NaOH) was used for redissolution of all samples and standards in order to apply an on-line preconcentration procedure based on Field Amplified Sample Stacking (FASS).

RESULTS AND DISCUSSION

Analysis of PSP Toxins

CE-UV detection was applied for the determination of the different PSP analogues, with the exception of the C toxin group because of their neutral charge in acidic media. This toxin group will co-migrate with electroosmotic flow. After optimisation of parameters affecting the separation and efficiency of PSP toxin analysis, such as pH, organic modifiers in the background buffer and voltage, good separation of these toxic compounds was achieved using the electrophoretic conditions described in the experimental section.

Enhanced sample loading and band stacking prior to zone electrophoresis was facilitated using a simple preconcentration procedure based on capillary isotachophoresis (CITP). Good efficiency and resolution in the separation of all the studied toxins, STX, dcSTX, NeoSTX and GTX group (GTX 1–5), were observed (Fig. 2A).

The isotachophoretic mode provided a significant increase in terms of detection limits for determining the presence of these PSP toxins in naturally contaminated samples. Calibration of the CE system was carried out by using individual standard solutions of STX and dcSTX, giving a linear relation between peak area and toxin concentration in a range between 0.1 and 10 μg/mL with $r^2 = 0.999$.

The method was applied to the analysis of PSP toxins present in naturally contaminated samples of Galician mussels. Good resolution was observed for the STX group, while analysis of the GTX group did not show a clear profile of the different GTX analogues involved in this contamination, as is shown in Fig. 2B. This low resolution could be attributed to the complexity of the matrix with its high salt content, as well as to the presence of influences and probably unknown GTX isomers. This last hypothesis has since been confirmed using MS detection [18].

The results obtained show the higher efficiency of CE when compared with HPLC as was expected from the features attributed to the electrophoretic technique.

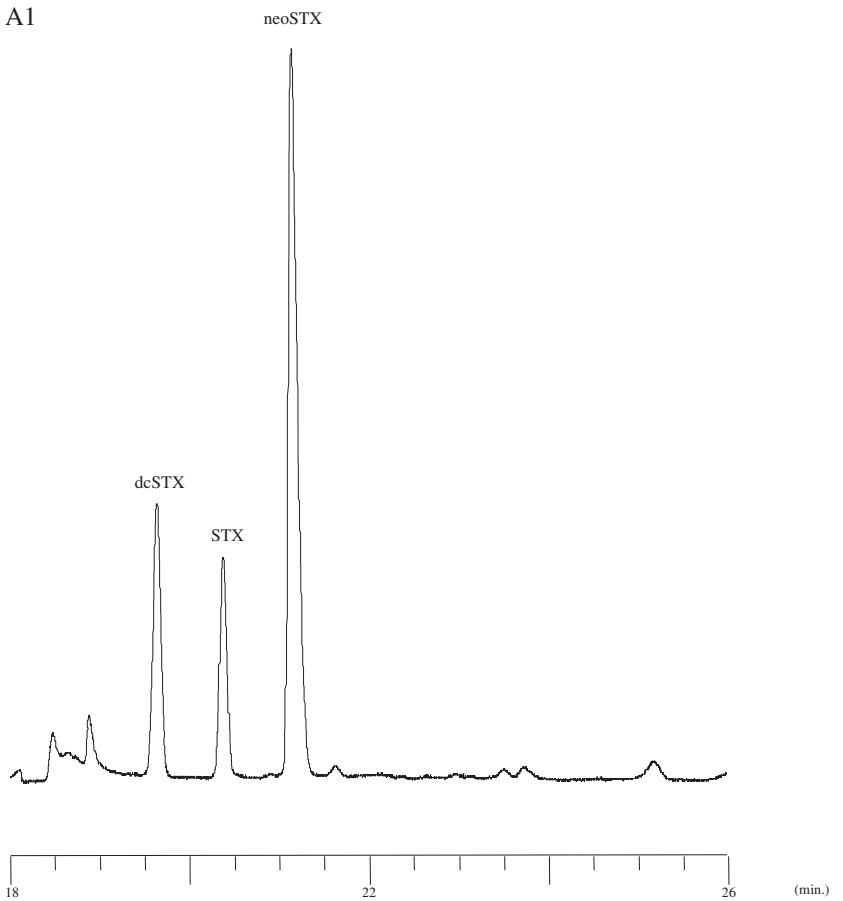


FIGURE 2 CE-UV/DAD analysis of: A PSP toxin standards; B contaminated Galician mussel sample extract (20 kV, 50-mM morpholine at pH 5.0, hydrodynamic injection 50 mbar, 90 s).

The simplicity of CE has also been shown, especially compared with the postcolumn HPLC-FLD method proposed by Oshima [10], where three different mobile phases had to be used for the isocratic elution of the three PSP groups while only a simple electrophoretic run is required for the analysis of all toxins involved in PSP toxicity with the exception of the C toxin group, which, owing to the overall neutral charge, are not able to be analysed by CE. Nevertheless, as was also expected, lower sensitivity was achieved for the CE analysis of these toxins, thus justifying the use of isotacophoresis as a procedure mode.

Analysis of ASP Toxins

The structure of domoic acid and analogues shows one amino and three carboxyl groups, which are responsible for five different charge states in this molecule at different pH values; therefore these toxins are capable of being determined by CE.

The CE conditions developed by Zhao *et al.* [11] with some modifications [16] were applied for ASP toxins, as was described in the experimental section. Borate under basic

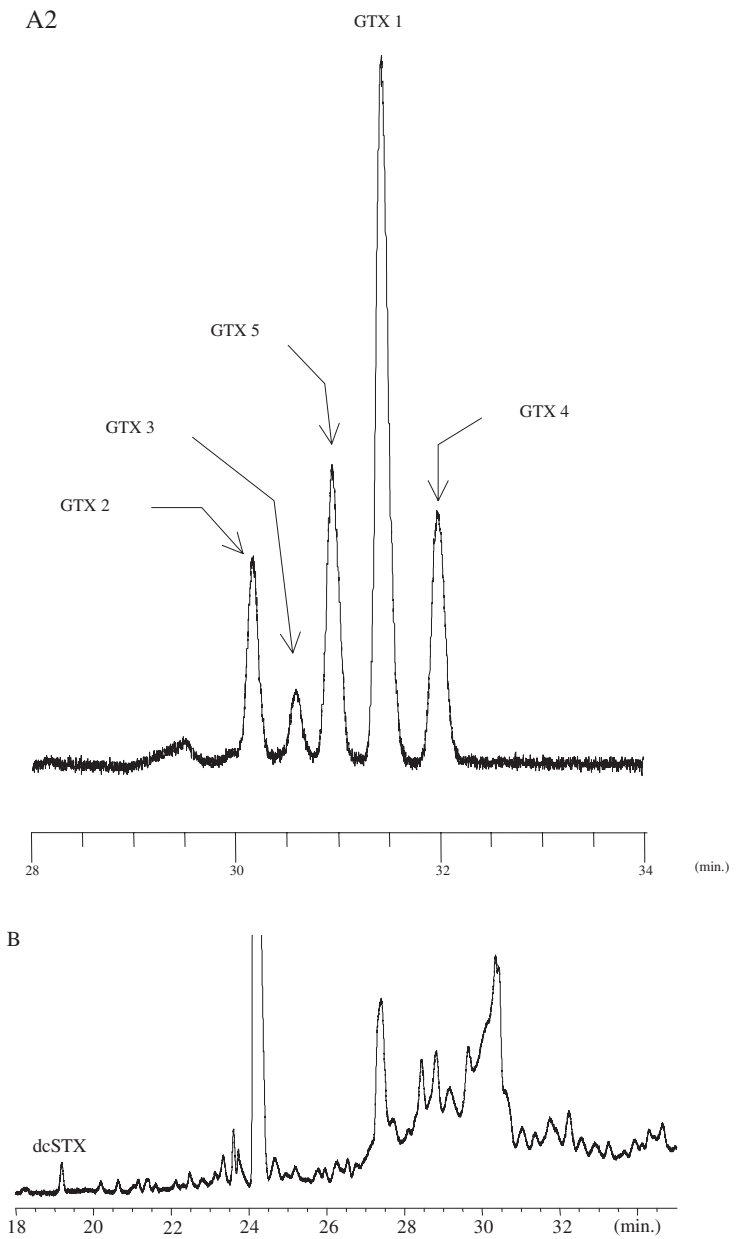


FIGURE 2 Continued.

conditions was the buffer chosen for the analysis and the ionic strength was selected as an optimisation parameter. Different borate buffer concentrations (10, 25 and 50 mM) were tested in order to find the optimal conditions in terms of efficiency and resolution for the separation of domoic acid and isomers. Both lower and higher concentrations affected the success of the analysis. In the case of 10-mM borate, the short migration times result in coelution with other compounds and domoic acid was not clearly

identified in naturally contaminated samples. An increase in the peak width was observed when 50-mM borate was used, increasing buffer diffusion in the capillary by the Joule heating effect. This lack of selectivity was overcome by using 25-mM borate which provided higher electrophoretic resolution and efficiency as a consequence of the sharpness of the peak corresponding to domoic acid. Calibration of the CE system was carried out for quantitative purposes by using standard solutions of domoic acid in a range 1.5–8 μg/mL with $r^2 = 0.998$.

The complexity of the matrix with significant presence of interfering compounds made the use of clean-up procedures strictly necessary prior to CE. These clean-up procedures also contribute to an increase of the sensitivity. SAX-SCX SPE procedure which had been initially described by Zhao *et al.* [11] has been evaluated in terms of recovery using MUS-1. Confirmation of the presence of domoic acid in the analysed samples was obtained by spiking samples with domoic acid standard solutions. Figure 3 shows examples of electropherograms corresponding to the analysis of extracts of MUS-1 and Galician razor clams.

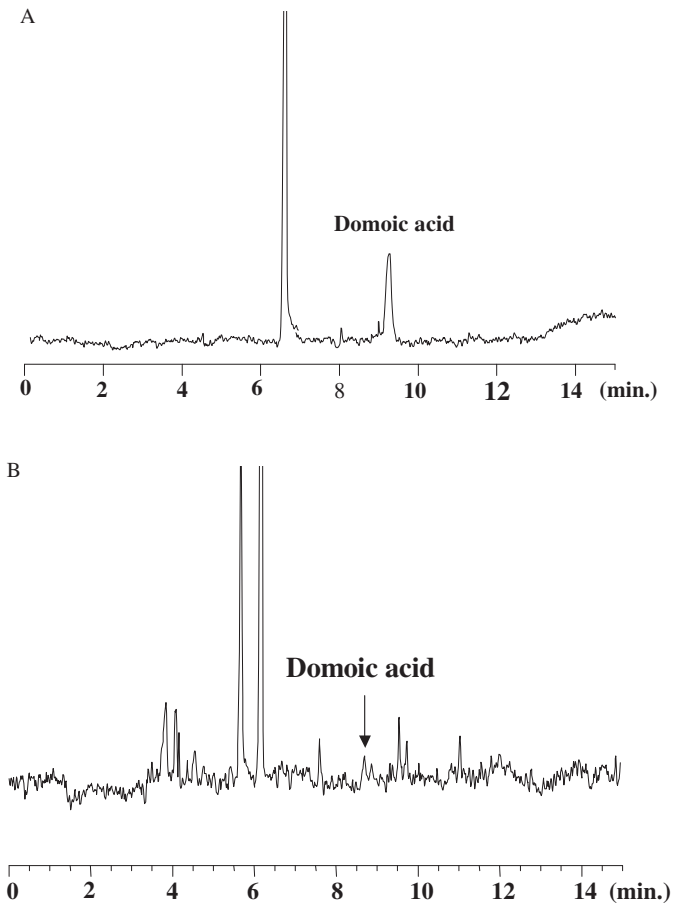


FIGURE 3 CE-UV/DAD analysis of: A Mussel Tissue Reference Material (MUS-1) and B Galician razor clam sample after SAX-SCX cleanup (30 kV, 25-mM borate at pH 9.2, hydrodynamic injection 50 mbar, 12 s).

The results obtained for the CE analysis of ASP, if compared with the ones usually obtained after conventional HPLC-UV, are in good agreement with those expected considering the main features of CE: higher efficiency and simplicity against a lower sensitivity; nevertheless, the sensitivity achieved in this particular case is good enough for the determination of the ASP toxins present in contaminated samples under the regulatory level, therefore CE is a promising technique for simple and fast monitoring of ASP toxins, although improvements in the sample clean-up are still required for selective removal of interferences as was observed for HPLC [19].

Analysis of YTX

CE in conventional operation mode, CZE, was applied for the analysis of YTX. Phosphate buffer at pH 8.5 with 20% MeOH as organic modifier was used as the background electrolyte. Two injection modes, electrokinetic and hydrodynamic, were compared in order to achieve the higher response of the CE system for the analysis of YTX. Parameters such as sensitivity, efficiency and analysis time were evaluated and 20 s of hydrodynamic injection at 50 mbar were the final optimised conditions. Calibration studies were carried out with YTX standards in a range 5–13.5 μg/mL obtaining $r^2 = 0.999$. Figure 4A shows an example of the results obtained from the electrophoretic analysis of a standard solution of YTX.

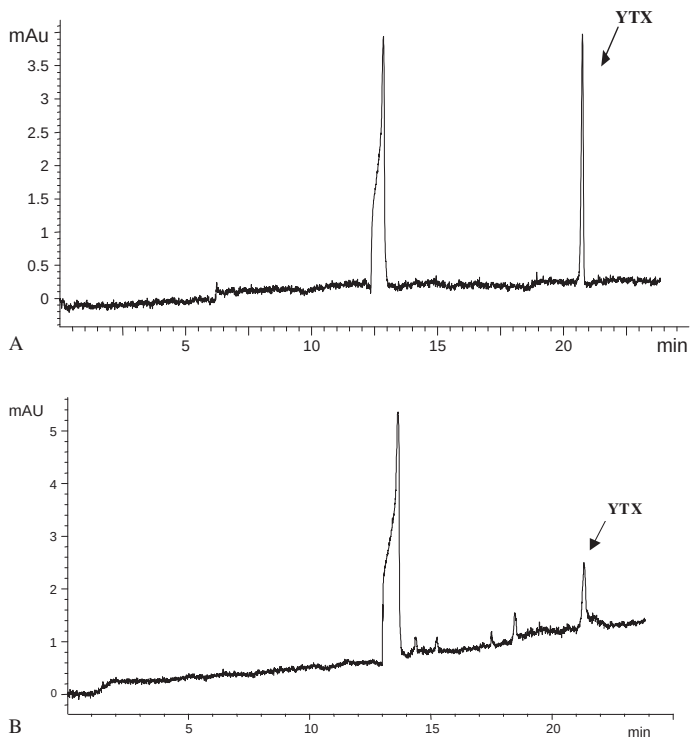


FIGURE 4 A Analysis of yessotoxin standard using CE-UV and B analysis of yessotoxin in natural contaminated sample using CE-UV/DAD (20 kV, 20% MeOH in 100-mM phosphate, pH 8.5, hydrodynamic injection 50 mbar, 20 s).

This method was applied for the analysis of green-shell mussels naturally contaminated with YTX (Fig. 4B). Sample pretreatment was carried out under the conditions described in the experimental section. The SPE procedure was critical to increase the concentration of YTX present in naturally contaminated samples to enable analysis by CE. After this concentration step the presence of YTX in those samples was determined and confirmation experiments were carried out by spiking sample extracts with standard solutions of YTX.

CE resulted in a very promising alternative for the analysis of YTX, particularly taking into account that the HPLC alternative that is commonly used for these particular toxins requires the use of complex derivatisation reagents that compromise the chromatographic performance. Nevertheless, improvements in the sensitivity of this technique are still required and different pre-concentration approaches are being investigated.

Analysis of Microcystins

Micellar electrokinetic chromatography (MEKC) resulted in an efficient approach for the analysis of microcystin toxins. MEKC combines the high efficiency of CE with the separation mechanism of chromatography because the micelles of surfactant act as a pseudo-stationary phase. The optimisation of parameters such as ionic strength and pH, as well as the SDS concentration in the buffer, was carried out based on peak areas, migration times, efficiency, effective mobility and capacity factor of the toxins and the current developed in the capillary. Values of 20–150 mM of borate and 10–50 mM of SDS in a range of pH from 5 to 9.5 were applied in the composition of the background electrolyte. Conditions of 120-mM borate with 20-mM SDS at pH 9.5 provided the overall best response. On-line preconcentration based on FASS was used for increasing the sensitivity achieved in the MEKC mode, and also contributed to an increase the efficiency in the electrophoretic separation. For the application of this stacking mode, samples were injected after being redissolved in a solution with lower conductivity, such as 30-mM borate following an optimisation procedure of Aguete *et al.* [20].

Figure 5A shows the separation of a standard mixture of three microcystins under the described conditions. These conditions were applied to the analysis of lyophilised material from a toxic bloom of cyanobacteria in freshwaters. The method allowed the determination of the named microcystins in naturally contaminated samples (Fig. 5B). Sample pretreatment was described in the experimental section. Quantitation of MC-LR, MC-YR and MC-RR was also possible after calibration of the system for the analysis of these toxins in the range 0.83–16.6 μg/mL with r^2 of 0.997, 0.998 and 0.999 respectively.

Detection and quantitation limits obtained for the studied toxins by CE method are shown in Table I.

Comparing results previously obtained in our laboratory for the HPLC-UV analysis of microcystins with these CE results, we conclude that higher efficiency and simplicity are achieved using CE, while higher sensitivity is achieved with HPLC, although the results obtained using both techniques were in a good agreement (data not shown), especially after using a suitable sample preparation as described in [20], which clearly contributed to an enhancement of the sensitivity of the CE approach.

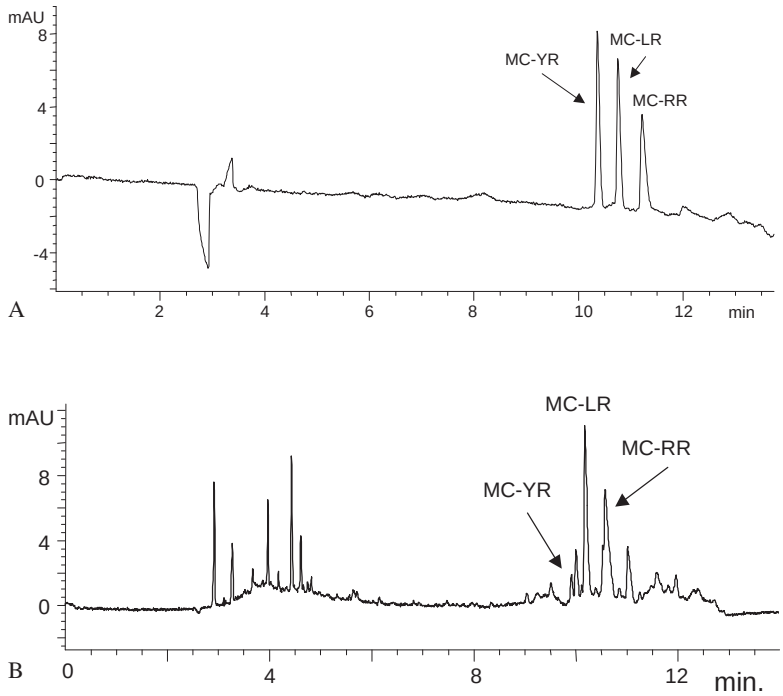


FIGURE 5 A Analysis of standard microcystins by CE-UV in MEKC mode. B Analysis of natural contaminated sample with microcystins after the clean-up procedure with SPE cartridges (25 kV, 20-mM SDS in 120-mM borate at pH 9.5, hydrodynamic injection 50 mbar, 25 s).

TABLE I Sensitivity parameters of CE methods evaluated for determination of aquatic toxins evaluated in terms of detection and quantitation limits

Toxins	*Compound*	D_L (μg/mL)	Q_L (μg/mL)
PSP	STX	0.05	0.17
	dcSTX	0.06	0.20
ASP	domoic acid	0.47	1.56
YTX	YTX	0.90	3.00
Microcystins	MC-YR	0.17	0.35
	MC-LR	0.20	0.46
	MC-RR	0.32	0.87

Conclusions

Suitable extraction and clean-up methods, based on SPE, contributed to efficient CE as an alternative for the analysis of naturally contaminated algal samples containing the named compounds.

The results obtained allowed us to conclude that CE provides a simple approach for an efficient and fast determination of the algal toxins described. The lack of sensitivity associated with CE has been overcome by using preconcentration techniques, or alternative CE modes such as isotacophoresis in the particular case of PSP toxins. An example of increased selectivity was also shown by using micellar electrokinetic chromatography for the CE analysis of MCs.

Acknowledgements

We thank Dr T. Yasumoto for providing the YTX standard solution as well as for the guidance in carrying out the CE analysis of YTX, also Dr Susana Franca from Instituto da Saúde Dr Ricardo Jorge for providing the cyanobacterial cells used in these studies. Financial support from the EU FEDER, Ref.: 1FD 97-0095 and Rampesca Fishing Co., as well as the Spanish Ministry of Education (CICYT, Ref: ALI96-2317) is also acknowledged. Technical assistance from David Calvar and A. Ulla is gratefully acknowledged.

References

[1] D.M. Anderson, J.J. Sullivan and B. Reguera, *Toxicon*, **27**, 665–674 (1989).
[2] S.S. Bates, C.J. Bird, A.S.W. deFreitas, R.A. Foxall, M.W. Gilgan, L.A. Hanic, G.E. Johnson, A.W. McCulloch, P. Odense, R. Pocklington, M.A. Quilliam, P.G. Sim, J.C. Smith, D.V. SubbaRao, E.C.D. Todd, J.A. Walter and J.L.C. Wright, *Can. J. Fish. Aqua. Sci.*, **46**, 1203–1215 (1989).
[3] H. Ogino, M. Kumagai and T. Yasumoto, *Nat. Toxins*, **5**, 255–259 (1997).
[4] W.W. Carmichael, *J. Appl. Bacteriol.*, **72**, 445–459 (1992).
[5] D.S. Smith and D.D. Kitts, *J. Agric. Food Chem.*, **43**, 367–371 (1995).
[6] C. MacKintosh, K.A. Beattie, S. Klumpp, P. Cohen and G.A Codd, *FEBS Lett.*, **264**, 187–192 (1990).
[7] M.A. Quilliam, P.G. Sim, A.W. McCulloch and A.G. McInnes, *Anal. Chem.*, **36**, 139–154 (1989).
[8] J.F. Lawrence, C. Ménard and C. Cleroux, *J. AOAC Int.*, **78**, 514–520 (1995).
[9] K.I. Harada, K. Matsuura, M. Suzuki, H. Oka, M.F. Watanabe, S. Oishi, A.M. Dahlem, V.R. Beasley and W.W. Carmichael, *J. Chromatogr.*, **448**, 275–283 (1988).
[10] Y. Oshima, *J. AOAC Int.*, **78**, 528–532 (1995).
[11] J.Y. Zhao, P. Thibault and M.A. Quilliam, *Electrophoresis*, **18**, 268–276 (1997).
[12] J. Wilson, R.W. Mark and R. Bruce, *J. High Resolut. Chromatogr.*, **20,** 34–38 (1997).
[13] *AOAC Official Methods of Analysis*, 15th edition, Method 959.08, pp. 881–882, Arlington, VA (USA) (1990).
[14] J.M. Leão, A. Gago Martínez, J.A. Rodríguez Vázquez, E.C. Aguete and M.M. Omil, *J. Chromatogr.* A, **798**, 131–136 (1998).
[15] S.J. Locke and P. Thibault, *Anal. Chem.*, **66**, 3436–3446 (1994).
[16] N. Piñeiro, J.M. Leão, A. Gago-Martínez and J.A. Rodríguez-Vázquez, *J. Chromatogr.* A, **847**, 223–232 (1999).
[17] K.P. Bateman, P. Thibault, D.J. Douglas and R.L. White, *J. Chromatogr.* A, **712**, 253–268 (1995).
[18] A. Gago-Martínez, J.A. Rodríguez-Vázquez, P. Thibault and M.A. Quilliam, *Nat. Toxins*, **4**, 72–79 (1996).
[19] A. Gago-Martínez, N. Piñeiro, E.C. Aguete, E. Vaquero, M. Nogueiras, J.M. Leão, J.A. Rodríguez-Vázquez and E. Dabek-Zlotorzynska, *J. Chromatogr.* A, **992**, 159–168 (2003).
[20] E.C. Aguete, A. Gago-Martínez, J.M. Leão, J.A. Rodríguez Vázquez, C. Menàrd and J.F. Lawrence, *Talanta*, **59**, 697–705 (2003).