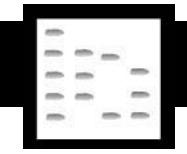
MARINE TOXINS: CHROMATOGRAPHY



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Introduction

The marine environment may be seriously affected by contamination due to the massive proliferation of toxic phytoplanktonic species for example toxic algae or 'algal blooms', which appear at certain times of the year under the influence of various environmental factors. These phytoplanktonic microorganisms are essential food for filter-feeding bivalve shellfish as well as other types of marine seafood. This phenomenon is commonly known as a 'red tide'.

These toxic algal blooms cause important socioeconomic damage to those regions that depend on aquaculture or fisheries industry, owing to their environmental and human health impacts.

It is believed that the first reference to a harmful algal bloom appears in the Bible:'… all the waters in the river turned to blood, the fish died'(Exodus 7:20–21). One of the first recorded fatal cases of human poisoning happened in 1793 in Poison Cove (British Columbia), caused by a group of alkaloids now called 'paralytic shellfish poisoning' toxins (PSP). Since this toxic event, a number of different toxic episodes have been reported in several places worldwide.

There are different type of shellfish poisoning: the main causative organisms and important toxicological effects are summarized in **Table 1**. In this article we will focus on the paralytic, diarrhoeic and amnesic shellfish poisoning toxing (PSP, DSP and ASP) because they are responsible for most of the toxic events on the European Atlantic Coast and are also of general occurrence in many other places worldwide.

These toxins cause important cause important human contamination and their impact has increased considerably over the last few years. For this reason, strict control of these toxins is necessary to prevent serious damage to health. The conventional mouse bioassay (still considered as official and routine methodology in most countries) is useful for determining most of the implicated toxins, but alternative analytical methods are desired, especially for research

Table 1 Main types of harmful algal blooms

Type of poisoning	*Causative organism*	*Symptoms*
Paralytic shellfish poisoning (PSP)	*Alexandrium catenella* *A. minutam* *A. tamarense* *Gymnodinium catenatum* *Pyrodinium bahamense*	Typical neurological symptoms. The severity of these symptoms is related to the ingested dose. Also symptoms like dizziness, nausea, vomiting, diarrhoea, muscular paralysis, respiratory diffculties, death through respiratory paralysis (in exteme cases).
Diarrhoeic shellfish poisoning (DSP)	*Dinophysis acuminata* *D. acuta* *D. fortii* *D. norvegica* *Prorocentrum lima*	Vomiting and diarrhoea typical of gastrointestinal disorders. Chronic exposure may cause tumour promotion, especially in the digestive system (stomach intestine and colon).
Amnesic shellfish poisoning (ASP)	*Pseudo-nitzschia multiseries* *Pseudo-nitzschia* *Pseudo-nitzschia austrials*	Typical gastrointestinal disorders (nausea, vomiting, diarrhoea). Neurological symptoms, especially in older people and people with chronic illnesses – confusion, short-term memory loss.
Neurotoxic shellfish poisoning (NSP)	*Gymnodinium breve*	Headache, diarrhoea, muscle weakness, nausea and vomiting. Paraesthesia, respiratory difficulties, vision alterations, speech difficulties.
Ciguatera poisoning	*Gambierdiscus toxicus* *Prorocentrum lima*	Gastrointestinal disorders (diarrhoea, abdominal pain, nausea, vomiting), difficulty in balance, heart problems (low heart rate and blood pressure), death through respiratory failure (in extreme cases).

purposes where high sensitivity and specificity are required.

Owing to the complexity of the sample matrix, separation techniques are required to remove interferences and to increase the selectivity in the analytical response. During the 1990s considerable research has been focused on the development of chromatographic approaches for the analysis of PSP, DSP and ASP toxins. Liquid chromatography (LC) has been shown to be one of the most successful alternatives for the sensitive determination of these compounds using different detection modes. Capillary electrophoresis (CE) is another analytical alternative that has been recently developed for the analysis of PSP and ASP toxins and, as we will discuss later, this is a promising technique for the analysis of such compounds.

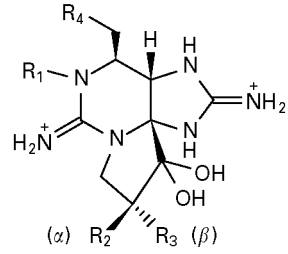
Separation Techniques for the Analysis of Marine Biotoxins

PSP Toxins

Paralytic shellfish poisoning (PSP) is a worldwide problem caused by consumption of shellfish that have accumulated potent neurotoxins produced by toxicogenic dinoflagellates. The PSP toxins include saxitoxin (STX) and several of its derivatives formed by addition of sulfo, hydrosulfate and *N*-1-hydroxyl groups (**Figure 1**).

The most common chemical method used for the analysis of PSP toxins is the combination of LC with online post-column oxidation and fluorescence detection, using two different isocratic and gradient elution modes. This evolved from earlier work, which showed that STX could be easily oxidized to a fluorescence derivative by hydrogen peroxide under alkaline conditions. Hydrogen peroxide is not able to oxidize the *N*-1-hydroxylated derivatives, which are better oxidized by using periodate. This reagent is therefore commonly used in post-column oxidation systems in order to detect all PSP toxins. LC combined with post-column oxidation has resulted in a successful approach but unfortunately is not without difficulties, especially concerning the operation of the equipment. This has made it necessary to optimize parameters such as stationary phase, mobile phase, etc. An alternative LC method employing prechromatographic oxidation has been reported. This method results in improved separation and quantitation of most PSP analogues. Modification of the periodate oxidation reaction for the *N*-hydroxy-containing toxins has led to improved sensitivity and stability of the products, enabling overnight analysis.

All the high performance liquid chromatography (HPLC) methods mentioned have resulted in valid approach for the control of these toxic compounds, but although these methods offer good sensitivity and dynamic range, the sensitivity is dependent on parameters such as reagent concentration, reaction times, pH and temperature of the oxidation reaction. In addition to the elaborate procedure required to achieve reliable and reproducible results, the main drawback of the alkaline oxidation reaction is the reliance on fluorescence response factors for the different PSP toxins based on the only commercially available standard. **Figure 2** shows an example of the



			Carbamoyl toxins	*N*-Sulfocarbamoyl toxins	Decarbamoyl toxins
R_1	R_2	R_3	R_4=OCONH$_2$	R_4=OCONHSO$_3^-$	R_4=OH
H	H	H	STX	GTX5	dcSTX
H	H	OSO$_3^-$	GTX2	C1	dcGTX2
H	OSO$_3^-$	H	GTX3	C2	dcGTX3
OH	H	H	neoSTX	GTX6	dcNEO
OH	H	OSO$_3^-$	GTX1	C3	dcGTX1
OH	OSO$_3^-$	H	GTX4	C4	dcGTX4

Figure 1 Chemical structure of PSP toxins.

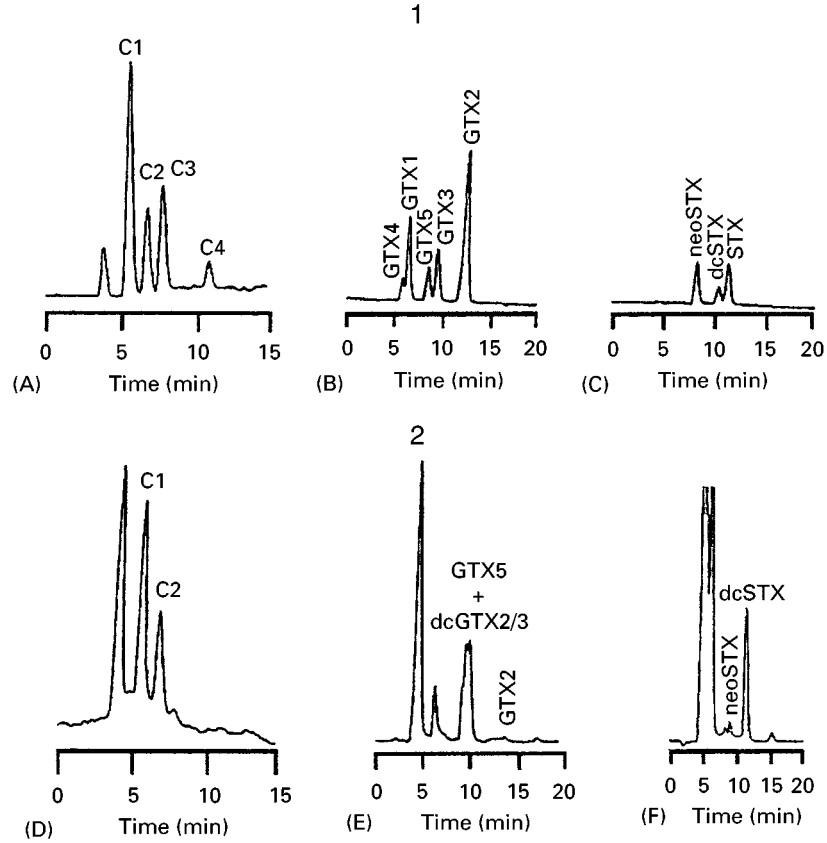


Figure 2 Chromatogram obtained for the PSP toxins profile by using post-column HPLC-FLD. (A) STX group; (B) GTX group; (C) C toxin group. 1, standard of PSP toxins; 2, mussel samples.

application of the LC technique with fluorescence detection for the analysis of PSP toxins in contaminated extracts of mussels for Galicia (northwest Spain). The conditions used to carry out this isocratic HPLC analysis with post-column oxidation are described in **Table 2**. Under these conditions good resolution was achieved for most of PSP toxins, with the exception of the gonyautoxin (GTX) group, owing to the presence of other GTX components with similar retention times (this was confirmed using mass spectrometric detection).

The successful separation of these toxins by both ion exchange chromatography and cellulose acetate electrophoresis prompted investigations of the application of CE to their analysis. With the exception of the Ciguatera (C) toxins, which have neutral overall charge, all PSP toxins have positive charge under acidic conditions, and can be separated by elec-

Table 2 Conditions for the post-column HPLC-FLD analysis of PSP toxins

HPLC instrument	Perkin-Elmer series 10-LC Column: reversed-phase, prodigy 5 μm C_8 Phenomenex 4.6 mm × 15 cm
Mobile phase	Flow rate, 0.8 mL min^{-1}
Mobil phase A (*for C toxin group*)	2 mmol L^{-1} tetrabutylammonium phosphate, pH 5.8
Mobile phase B (*for GTX toxin group*)	2 mmol L^{-1} sodium 1-heptanesulfonate in 10 mmol L^{-1} ammonium phosphate, pH 7.3
Mobile phase C (*for STX group*)	2 mmol L^{-1} sodium 1-heptanesulfonate in 30 mmol L^{-1} ammonium phosphate, pH 7.1, 5% v/v acetonitrile
Oxidizing reagent	7 mmol $^{-1}$ potassium periodate in 50 mmol L^{-1} potassium phosphate buffer, pH 9.0; flow rate: 0.4 mL min^{-1}
Reaction system	In 10 m Teflon tubing (0.5 mm i.d.) at 65°C in water bath
Acid solution	0.5 mol L^{-1} acetic acid; flow rate: 0.4 mL min^{-1}
Detection	Hitachi F1000 fluorescence detector, double monochromator. Excitation wavelength 330 nm; emission wavelength 390 nm

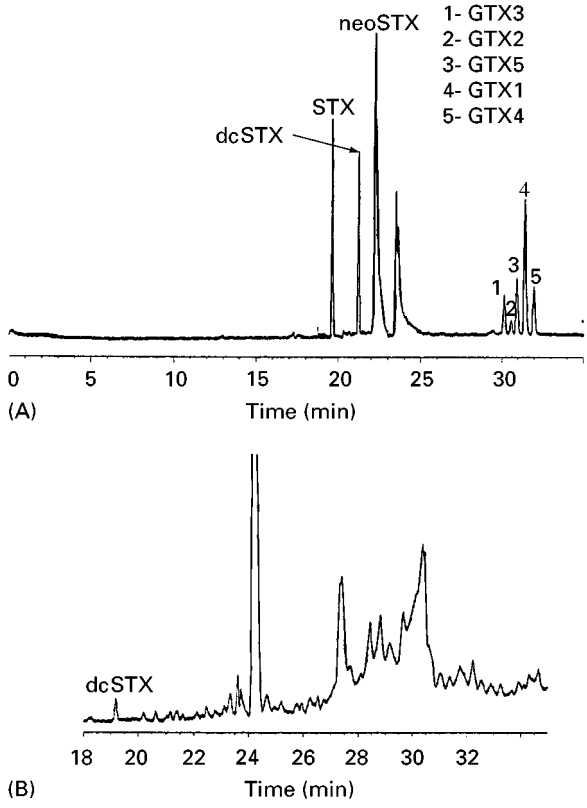


Figure 3 Electropherogram obtained for the PSP toxins profile by using CE-UV-DAD. (A) standard of PSP toxins; (B) mussel sample.

trophoresis. Capillary electrophoresis should provide an efficient separation of most PSP components; however, some of the inherent difficulties in analysing these compounds are the lack of a chromophore absorbing in the usual UV range as well as the lack of standards to confirm the electrophoretic peak identity.

To overcome situations where the electrophoretic peak identity presents some uncertainties, or standards are not available to correlate with the peaks of interest, it is necessary to use a complementary technique, such as mass spectrometric detection using electrospray ionization. This technique has shown excellent sensitivity for PSP as well as for other marine toxins. Mass spectrometric detection coupled with CE has been used for the rapid and efficient determination of PSP toxins. This technique has also been applied for the analysis of real mussel samples and an efficient separation for most of PSP toxins was achieved.

An example of the application of CE to the analysis of PSP toxins in mussels is shown in **Figure 3**. Under the conditions described in **Table 3**, several contaminated Galician mussel samples were analysed by this technique. Clean-up of the samples was required to remove interferences, but after this clean-up good resolution was obtained for most PSP toxins with the exception of the C toxins, which are not ionized in acidic media. The potential of this technique in terms of sensitivity was clearly increased by using isotacophoresis. Resolution in terms of efficiency, by means of theoretical plates, was clearly higher than that achieved by using HPLC.

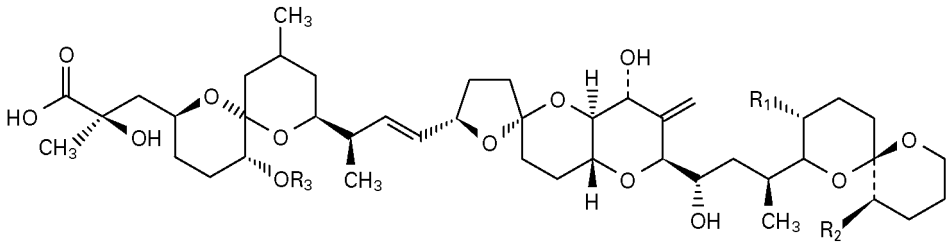
DSP Toxins

Since PSP toxins are potent neurotoxins, the term '*diarrhoeic shellfish poisoning*' (DSP) has been associated with a number of different groups of toxic compounds; these include polyether compounds such as okadaic acid (OA), dinophysistoxins (DTX1, DTX2, DTX3), pectenotoxins and the fused polyether yessotoxin. Toxicological studies have shown that okadaic acid and dinophysis compounds are potent phosphatase inhibitors; their common symptomatology is related with the occurrence of diarrhoea. The mechanism of action of the other compounds has not been fully established, but it seems that they do not cause diarrhoea; instead they are described as hepatotoxins. The main reason for including these toxins in the DSP group is probably their polyether structure (**Figure 4**).

DSP toxins are also produced by certain toxic dinoflagellates; the chemical structure of these toxins emerged following the isolation of new polyether toxin named okadaic acid, from the sponge *Hali-*

Table 3 Conditions for the CE-UV-DAD analysis of PSP toxins

CE-UVD System	HP ^{3D}CE (Hewlett-Packard); voltage 20 kV
Capillary	Polyvinyl alcohol (PVA) capillary (75 μm i.d. and 104 cm length)
Background buffer	50 mmol L^{-1} morpholine in water adjusted at pH 5 with formic acid
Injection	Sample injection into capillary was 20% of capillary volume. Pressure, 50 mbar; time, 120 s
ICTP	Voltage, 20 kV Leading buffer, 10 mmol L^{-1} formic acid Terminating buffer, 50 mmol L^{-1} morpholine in water adjusted at pH 5.0 with formic acid Time, 90 s
UVD detection	Wavelength 200 nm



R_1	R_2	R_3	
CH_3	H	H	Okadaic acid (OA)
CH_3	CH_3	H	Dinophysistoxin-1 (DTX1)
H	CH_3	H	Dinophysistoxin-2 (DTX2)
H / CH_3	H / CH_3	Acil	Dinophysistoxin-3 (DTX3)

Figure 4 Chemical structure of DSP toxins.

chondria okadai. The similarities between okadaic acid and DTXs were quickly recognized. Several places worldwide have been affected by such toxic outbreaks since the first toxic event, which took place in the Netherlands in 1960. Although the toxic effects of okadaic acid are related to gastrointestinal disorders, these toxins have been shown to have the potential to bind to protein phosphatases. Consequently the toxicological activity of DSP toxins is also associated with the promotion of tumours, especially in the stomach, intestine and colon.

As for PSP toxins, mouse bioassay is the method commonly used for the analysis of DSP compounds, but the problems associated with this bioassay, such as long assay time, poor reproducibility and false positives, have instigated the search for alternative techniques. Since DSP toxins are lipid-soluble, organic solvents are required for their extraction. However, such lipid-soluble extracts are considerably more complex than aqueous extracts of the same organism and for this reason additional clean-up steps are required before analysis. DSP toxins can be detected by thin-layer chromatography (TLC), although the presence of interferences causes difficulties when using this technique.

DSP toxins may be separated by reversed-phase HPLC using an octadecylsilica (ODS) stationary phase and an acidified aqueous acetonitrile or methanol mobile phase. Detection can be accomplished with UV absorbance at 205–215 nm or with a refractive index detector, to give a detection level of about 10 $\mu g\,mL^{-1}$ in solution, but the low selectivity of these detectors requires a high degree of clean-up prior to analysis. DSP toxins have a carboxyl group that is easily converted into a fluorescent ester derivative, which allows HPLC analysis with fluorescence detection. Pioneering work using this fluorescence detection method uses 9-anthryldiazomethane (ADAM) as the derivatization reagent. Several other reagents have also been used for derivatization to try to overcome the problems of instability of ADAM; however, none has proven as selective and sensitive as ADAM. An HPLC method has been developed with fluorescence detection, using ADAM as derivatization reagent, which was synthesized *in situ* and used immediately. This method offers a reformulation of the previous *in situ* method. The ADAM method is very sensitive for DSP toxins, being able to detect 10 pg of the okadaic acid (OA) derivative injected on-column; the practical quantitation limit is about 10 $ng\,g^{-1}$ tissue. **Figure 5** shows an example of the application of the *in situ* ADAM-HPLC analysis of standards and a real Galician mussel sample under the chromatographic conditions described in **Table 4**. This ADAM method is not suitable for the analysis of DTX-3 compounds owing to their high molecular weight and lipophilicity. They must first be converted back to OA, DTX-1 or DTX-2 via alkaline hydrolysis; nevertheless, these compounds can be directly analysed by mass spectrometric techniques.

Mass spectrometry is a powerful tool for the analysis of marine toxins. This technique can provide structural information, as well as offering high sensitivity and selectivity; this structural information is useful not only for the confirmation of toxin identity, but also for the identification of new toxins. The combination of HPLC with electrospray mass spectrometry (LC-ESMS) appears to be one of the most sensitive and rapid methods of analysis for DSP toxins. The detection limit found for this technique is about 1 $ng\,g^{-1}$ in whole edible shellfish tissue. This mass spectrometric detection has been also applied for the analysis of Galician samples, allowing the first confirmation of DTX-2 in these mussels.

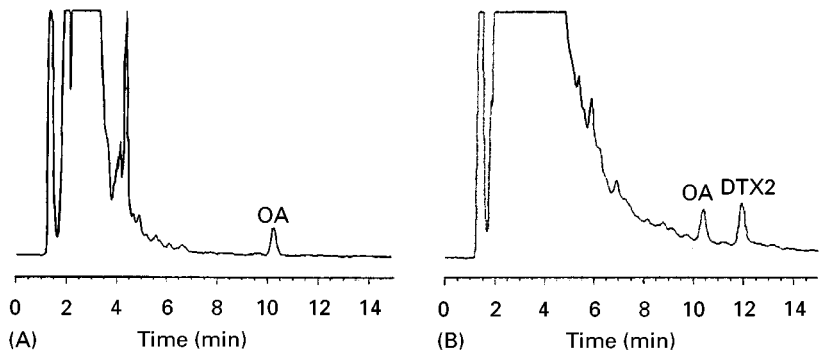


Figure 5 Chromatogram obtained for the DSP toxin profile by using ADAM-HPLC-FLD.

ASP Toxins

This new type of seafood toxicity was first described after a contamination that took place in Prince Edward Island, Canada, in 1987. None of the known shellfish toxins was implicated in this incident and eventually domoic acid was identified as the toxic agent. Amnesic shellfish poisoning was originally isolated from a red macroalga, *Chondria armata*, by Japanese researchers studying insecticidal properties of algal extracts. Most of the people affected by this intoxication experience gastroenteritis but many older people develop neurological symptoms including memory loss. Intraperitoneal injections of acidic aqueous extracts of mussels contaminated with domoic acid into mice cause death with unusual neurotoxic symptoms very different from those of paralytic shellfish poison and other known toxins.

Domoic acid (DA) is a known neurotoxin that is absorbed through the gastrointestinal system, causing damage in the central nervous system. The source of this toxin is a diatom, *Nitzschia pungens multiseries*, which is ingested by shellfish such as mussels during normal filter feeding. The chemical structure of domoic acid is shown in **Figure 6**. This rare naturally occurring amino acid is a member of a group of potent neurotoxic amino acids that act as an agonist to glutamate, a neurotransmitter in the central nervous system. A number of DA isomers that show varying degrees of toxicity have also been identified. Isomerization of DA can occur photochemically or thermally, the latter being significant in cooked seafood.

Like the toxins previously described, reliable methods for the analysis of DA and isomers in seafood products are extremely important for protection of public health. Domoic acid can be analysed semiquantitatively by TLC, but instrumental methods of analysis are most commonly used. HPLC or ion exchange chromatography using ultraviolet absorbance detection are the methods of choice. HPLC has been used since 1987 by Canadian regulatory agencies to prevent other incidents of shellfish poisoning, and is also the official method of analysis for domoic acid in most countries. Domoic acid may be extracted from shellfish tissues by boiling with water or by blending with aqueous methanol. The latter is most commonly used because it is better suited for trace analysis and combines well with a highly selective clean-up based on strong anion exchange. The detection of domoic acid is facilitated by its strong absorbance at 242 nm. The main problem of using this technique is associated with the presence of interferences, which can given false positives with crude extracts. This is the case of tryptophan and some of its derivatives. Since these compounds are often present in shellfish and elute close to domoic acid, SAX-SPE clean-up prior to HPLC-UV analysis avoids the problem caused by these

Table 4 Conditions for the ADAM-HPLC-FLD analysis of DSP toxins

HPLC system	Liquid chromatograph, HP-1050
Column	Reversed-phase column, HP-Hypersil ODS (4 mm i.d. $\times$ 25 cm, 5 μm)
Mobile phase	MeCN : H_2O (85 : 15)
Flow rate	1.0 mL min^{-1}
Detection	Fluorescence detector, HP-1046A: excitation wavelength 254 nm emission wavelength 412 nm

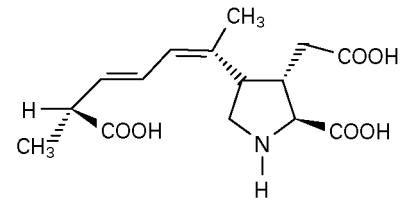


Figure 6 Chemical structure of domoic acid, main toxin responsible for ASP toxicity.

Table 5 Conditions for the HPLC-UV analysis of ASP toxins

HPLC system	Liquid chromatograph Jasco PU-980 pump
Column	Prodigy-ODS 5 μm column (Phenomenex) 4.6 mm × 25 cm
Mobile phase	10% v/v aqueous acetonitrile with 0.2 mol L^{-1} formic acid; flow rate, 1 mL min^{-1}
UV detector	Perkin-Elmer LC-95 UV/Vis; wavelength 242 nm

interferences. An example of the application of the HPLC-UV technique for the analysis of domoic acid in standards, mussel tissue reference material and real contaminated bivalves under the chromatograhic conditions described in **Table 5** in shown in **Figure 7**.

A very sensitive alternative HPLC method using fluorescent detection has been proposed. This method uses 9-fluoronylmethyl chloroformate (FMOC) as derivatization reagent and the FMOC derivative is analysed with fluorescence detection.

Capillary electrophoresis offers the potential of fast high resolution separation of DA and its isomers and the possibility of trace analysis with very small amounts of sample. This method is also attractive in terms of being inexpensive and complementary to HPLC.

Extraction and clean-up procedures are priority steps in order to achieve the best chromatographic and electrophoretic resolution; when combined with a selective extraction and clean-up procedure, CE with UV detection is an excellent method for the separation and quantitative analysis of domoic acid and its isomers in shellfish tissues. **Figure 8** shows the

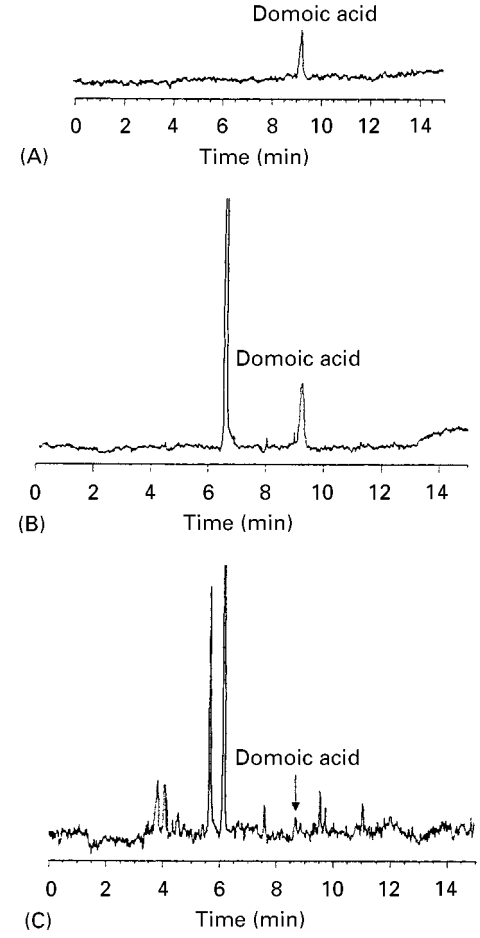


Figure 8 Electropherogram obtained for domoic acid by CE-UV-DAD. (A) Standard of domoic acid; (B) mussel tissue reference material (MUS-1); and (C) Galician mussel sample. (Reproduced with permission from James KJ *et al. Journal of Chromatography* 798: 147.)

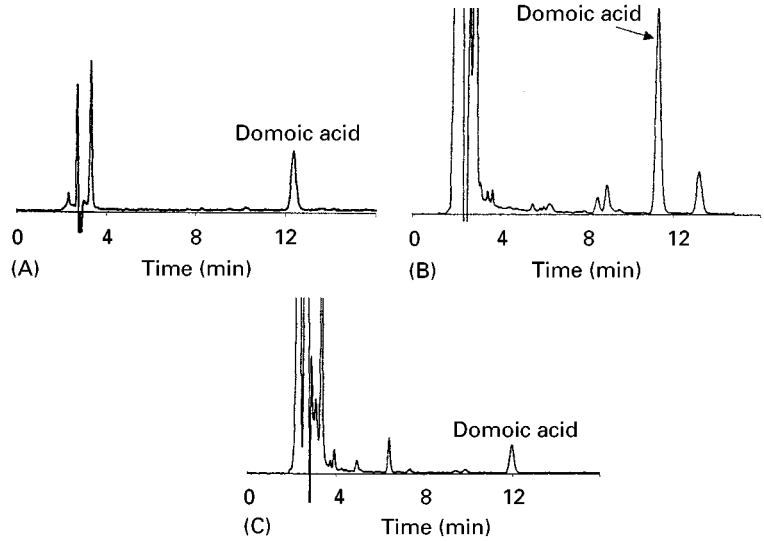


Figure 7 Chromatogram obtained for domoic acid by HPLC-UV. (A) Standard of domoic acid; (B) mussel tissue reference material (MUS-1); and (C) Galician mussel sample.

Table 6 Conditions for the CE-UV-DAD analysis of domoic acid

CE-DAD system	HP ^{3D}CE (Hewlett-Packard); voltage, 30 kV
Capillary	Bare fused silica capillary (50 μm i.d. × 66 cm length)
Background buffer	25 mmol L^{-1} borate at pH 9.2
DAD detector	Wavelength 242 nm
Injection	Pressure 50 mbar; time, 12 s

application of CE to the analysis of standard solution of domoic acid, mussel tissue reference material (MUS-1) and Galician mussel samples. The conditions for this CE analysis are summarized in **Table 6**.

The use of cyclodextrins allows an increase on the separation efficiency when compared with LC-UVD. The better mass detection limit provided by this CE technique could be very useful for situations where limited sample size is available.

Future Trends

The techniques of HPLC and CE for the analysis of marine toxins provide the separation efficiency required for complicated matrices such as marine samples as well as the high resolution required to determine the toxins present in contaminated samples with very low detection limits. Extraction and clean-up steps prior to the chromatographic analysis are essential in order to obtain accurate quantitative results and also to prevent interferences that can cause false positives. New, fast and on-line automated procedure could give shorter and more accurate analyses. The possibility to combine this automation with simple methods is also desirable, especially for routine control purposes.

Taking into account the lack of standards for all toxins and also the appearance of new, unknown toxic compounds, the development of confirmatory techniques for their detection is an important research field. The development and optimization of coupling techniques, such as LC-MS or CE-MS, and the development of adequate interfaces as well as efficient ionization modes is a high priority.

See Colour Plate 104.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry; Instrumentation; Mechanisms: Reversed Phases. **Electrophoresis:** Capillary Electrophoresis; Capillary Electrophoresis-Mass Spectrometry.

Further Reading

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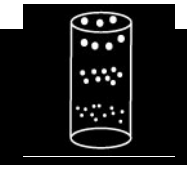
MECHANICAL TECHNIQUES: PARTICLE SIZE SEPARATION



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Introduction

Particles of many kinds and various sizes have played an important role in man's interaction with his physical environment. They abound in the soil and earth below; they are also present in water, air, chemical products, and many other sources. If particles were spherical or cubical, it would be easy to characterize them. Unfortunately, most of the particles present in our environment are of irregular size and shape. Therefore, it is desirable to try to develop methodologies and techniques to characterize particles of irregular size and shape, and this is the main objective of particle size analysis. Moreover, particle size analysis is important in studying particle behaviour in a medium as in many analytical sciences and industrial applications.

Particle size analysis in physical, chemical, and biological processes involves many concepts and techniques; however, this article focuses on the methods of particle size analysis utilizing mechanical techniques.

This article will first introduce some basic principles used in particle size analysis. This will be followed by a summary of the applicable particle size ranges for the different methods and the size ranges of most common particles found in industrial, chemical, environmental, and clinical applications. The most common mechanical techniques and methods used in particle size analysis will be briefly presented.

Particle Properties

Particle size analysis plays an important role in many analytical sciences and industrial applications. To assist in developing useful methodologies and techniques it is essential to identify the main factors that control the behaviour of particles in a medium. Such factors include particle density, shape, size, size distribution, concentration, and surface characteristics, and the carrier medium dynamics (**Figure 1**). This article focuses on particle size analysis using mechanical techniques in relation to clinical, industrial, and environmental applications: therefore, the particles under consideration could be solid or liquid and the medium could be liquid or gas. In aerosol systems the medium is gas (air).

Density

Particles originating from a solid will have the same density as that of the parent material. However, if the material undergoes hydration or surface oxidization or if it agglomerates in clusters, its specific gravity will change. The particle density plays an important role in the separation of solids as in centrifugal and gravitational sedimentation, for example.

Particle Shape

Shape factors The method of formation influences the resultant particle shape. In comminution, attrition or disintegration, the generated particle resembles the parent material. On the other hand, if the method of formation is condensation from vapour, the smallest unitary particle may be spherical or cubical. In many cases condensation is followed immediately by solidi-

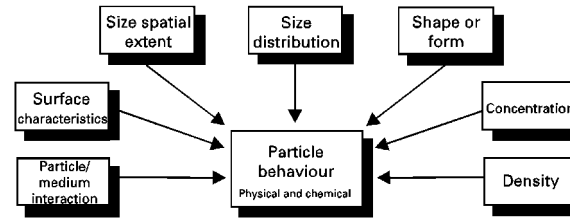

Figure 1 Main factors affecting particle behaviour in a medium.