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Comparison of liquid-junction and coaxial interfaces for capillary electrophoresis–mass spectrometry with application to compounds of concern to the aquaculture industry[☆,☆☆]

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ABSTRACT

The application of capillary electrophoresis–mass spectrometry (CE–MS) to the analysis of compounds of concern to the aquaculture industry is reported. Two different approaches to coupling the CE column to an IonSpray atmospheric pressure ionization (API) interface, *viz.*, a liquid-junction and a coaxial arrangement, are described and compared with regard to ruggedness, ease of use, sensitivity and electrophoretic performance. The different injection modes used in three commercial capillary electrophoresis systems were also evaluated for their applicability to CE–MS. The use of CE–MS for the analysis of a variety of classes of antibiotics used in the fish aquaculture industry, such as the sulfonamides and their potentiators (*e.g.*, trimethoprim), is demonstrated and was used to confirm the presence of these components in shellfish extracts at the low ppm level. CE–MS was also applied to the analysis of marine toxins such as saxitoxin and its analogues which are associated with paralytic shellfish poisoning, and also to the toxins responsible for amnesic and diarrhetic shellfish poisoning. Tandem mass spectrometry (MS–MS) was used to provide structural information on these analytes, and the ability to distinguish isomeric compounds based on their different migration and fragmentation characteristics using CE–MS–MS is demonstrated.

INTRODUCTION

The last decade has seen a dramatic rise in aquaculture operations in Canadian coastal waters. As in terrestrial agriculture, intensive farming techniques have resulted in the increasing use of antibiotics to control disease. A major concern with the use of these compounds is that residues may be present in food products if proper withdrawal times for treated animals have not been strictly observed. The tolerance levels established by regulatory agencies (typically 0.1 ppm) demand the development of highly sensitive and selective confirmatory methods for these compounds in marine species and other food products.

As well as coping with the problems of man-made chemicals, there are other natural hazards to the particularly fragile shellfish industry. Toxic algal blooms occur all over the world and appear to be increasing in number and diversity [1]. Of major concern are the species of phytoplankton whose metabolites can cause human illness ranging from mild discomfort to paralysis, and even death, following the ingestion of shellfish which have been feeding on the toxic algae. The most notorious hazard in North America is paralytic shellfish poisoning (PSP), caused by a group of highly potent neu-

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rotoxins produced by the "red-tide" dinoflagellates, *Alexandrium* spp. [2]. Other toxins of concern to the Canadian shellfish industry include those responsible for amnesic shellfish poisoning (ASP, [3]) and diarrhetic shellfish poisoning (DSP, [4]).

Although high-performance liquid chromatography (HPLC) is currently the most commonly used instrumental technique for the analysis of both antibiotics [5,6] and marine toxins [7–9], capillary electrophoresis (CE), initially described by Jorgenson and Lukacs in 1981 [10], is rapidly becoming an important analytical tool, complementary to HPLC [11]. The technique exploits differences in electrophoretic mobilities of charged compounds under an applied electric field, and is characterized by extremely high separation efficiencies. Both HPLC and CE, however, are often frustrated by the lack of a sensitive, universal detector. The most common detection systems used with CE thus far have been ultraviolet (UV) and fluorescence spectrophotometry [12], although electrochemical detection has also been reported [13]. In view of the potential of coupling high-resolution separation techniques with mass spectrometry as the ideal confirmatory method, we have undertaken to develop such methods for the analysis of compounds of concern to the aquaculture industry. We have previously demonstrated the capability of LC–mass spectrometry (LC–MS) and LC–tandem MS (LC–MS–MS) to provide confirmatory analysis of both antibiotics in fin-fish [14] and marine toxins in phytoplankton [15].

Other workers have reported the successful coupling of CE to MS using either atmospheric pressure ionization (API) techniques such as electrospray (ES) [16–19] and ionspray (ISP) [20,21], or fast atom bombardment ionization (FAB) using a continuous-flow (CF-FAB) interface [22–25]. With both ionization techniques, two approaches to interfacing CE to mass spectrometry have emerged. The first uses what is termed a "liquid-junction" [20–22,25], in which the CE column is connected, via a tee-junction, to a transfer line which is in turn coupled to the ion source of the mass spectrometer. A make-up flow of a suitable buffer (containing a matrix such as glycerol in the case of CF-FAB) is added at the liquid junction. The other approach employs a "coaxial" capillary arrangement [16–19,23,24] with the CE column inside a sheath capillary through which the make-up buffer is added concentrically. In this configuration the CE column terminates at the ion source. Both approaches have succesfully demonstrated the potential of CE–MS for the analysis of drugs [25], amines [18], sulfonated azo dyes [21], quaternary phosphonium [18] and ammonium salts [16,17] and peptides and proteins [19,20,22–25]. More recently, a CE method with UV detection for the determination of underivatized PSP toxins was reported [26], in which a preliminary CE–MS interface was used to confirm UV peak identities.

In this paper we describe the design and application of two robust, interchangeable interfaces for CE–MS utilizing ISP on a triple quadrupole mass spectrometer. The practical details of their construction and use are discussed, together with the advantages and disadvantages of the various injection modes employed on commercial capillary electrophoresis systems. We have developed CE methods for a variety of classes of antibiotics used in aquaculture, including the sulfonamides and their potentiators (*e.g.*, trimethoprim), tetracyclines and macrolide antibiotics such as erythromycin, as well as for the analysis of saxitoxin and other PSP toxins in phytoplankton and shellfish extracts. MS–MS has been used to differentiate isomeric compounds and provide confirmatory analysis using selected reaction monitoring. The application of CE–MS to other types of marine toxins, such as those responsible for amnesic (ASP) and diarrhetic shellfish poisoning (DSP), is also reported.

EXPERIMENTAL

Chemicals

All of the antibiotics used were obtained from Sigma (St. Louis, MO, USA). Domoic acid is supplied as an instrument calibration solution (DACS-1) from this laboratory through the Marine Analytical Chemistry Standards Program. Sodium hydroxide (AnalaR grade) was obtained from BDH (Toronto, Canada). Acetonitrile was Fisher Scientific OPTIMA grade, and HPLC-grade water was generated from glass-distilled water using a Millipore Milli-Q Reagent Water System. Trisma buffer (pH 7.2) was prepared by appropriate additions of Trisma HCl (*ca.* 0.7 g) and Trisma base (*ca.* 0.07 g) in 100 ml of distilled water. Cyclohexylaminopropyl

sulfate (CAPS) buffer (pH 11) was obtained from Applied Biosystems (ABI, San Jose, CA, USA).

Biological extracts

Shellfish (oyster) tissue fortified with known levels of antibiotics was extracted using procedures described previously [14]. Saxitoxin (STX) and other PSP toxins were isolated from a toxic strain of *Alexandrium excavatum* dinoflagellates as described previously [26]. Okadaic acid was isolated from a culture of *Prorocentrum lima* using a combination of column chromatography and preparative HPLC [27].

Capillary electrophoresis

Three commercially available capillary electrophoresis systems were used: a ISCO (Lincoln, NE, USA) Model 3850, a P/ACE System 2100 (Beckman Canada, Mississauga, Canada) and an Applied Biosystems (ABI) Model 270A. All three systems were equipped with variable-wavelength UV detectors using through-column optics. Both the ISCO and ABI systems were interfaced, via a Hewlett-Packard Model 3396A integrator, to an MS-DOS computer based on a 80286 processor using Chromperfect software (Justice Innovations, Palo Alto, CA, USA). The Beckman system was interfaced directly to an MS-DOS computer (80386 processor) using a Beckman System Gold dedicated software package. The ISCO system was equipped with a manual split-flow injector, which utilizes PEEK tubing of various lengths and/or I.D. as a restrictor to vary the splitting ratio. The ABI and Beckman systems both employ software-controlled hydrodynamic injection techniques in which either a vacuum is applied at the detector end reservoir (ABI), or pressure is applied at the injection end reservoir (Beckman). All three systems are also capable of electrokinetic injections, although the ISCO system had to be modified in-house to make this possible. The advantages and disadvantages of the various injection modes, with regard to coupling the electropherographs to atmospheric pressure ionization mass spectrometry (API-MS), will be discussed later. All untreated fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA) and CE separations were achieved on columns of 50 μm I.D. and between 75 and 90 cm in length.

Mass spectrometry

All combined CE–MS and CE–MS–MS experiments were performed on a SCIEX (Thornhill, Canada) API III triple quadrupole mass spectrometer equipped with an API source. A Macintosh IIx computer was used for instrument control, data acquisition and data processing. The two CE–MS interface configurations used were both constructed from a commercial fully-articulated IonSpray (ISP) interface (SCIEX) (Fig. 1). Details of the modifications are described below. Aqueous formic acid (0.2%) was used as the make-up flow in both configurations for positive ion analyses. In the liquid-junction configuration the make-up reservoir consisted of a 1-ml syringe mounted on the ISP interface flange, whereas in the coaxial design the make-up was delivered at 3–8 μl/min by a Microgradient LC syringe pump (ABI) via a submicroliter injection valve (Valco) with a 60-nl loop. This injector was used to optimize the interface and for all flow-injection experiments. The voltage on the ISP interface was maintained at *ca.* 5.6 kV, *i.e.*, providing an effective 24.4 kV across the CE column when

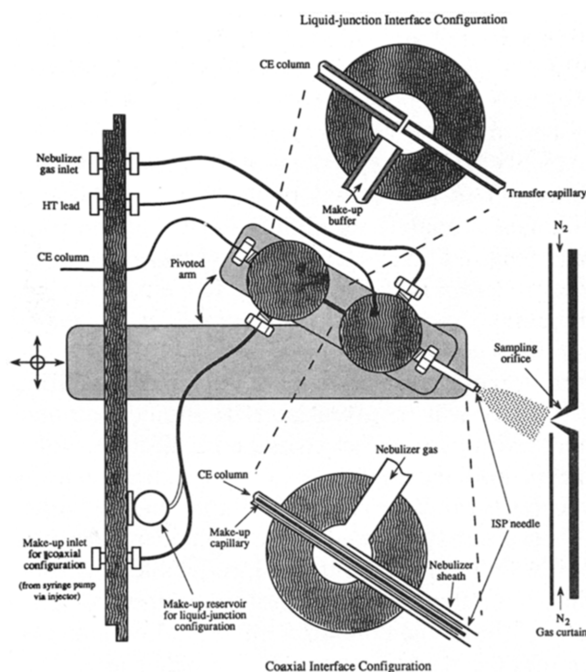

Fig. 1. Schematic diagram of the fully-articulated IonSpray interface showing the liquid-junction and coaxial configurations for CE–MS.

operating in the positive ion mode with 30 kV applied at the cathode. High-purity air was used as the nebulizing gas. Dwell times of 5 and 200 ms per dalton were used for full-scan and selected ion monitoring LC–MS experiments, respectively. MS–MS measurements were based on collision-induced dissociation (CID) of protonated molecules in the RF-only quadrupole using a collision energy of 20–35 eV and a target gas thickness (argon) of $3.0 \cdot 10^{14}$ molecules cm^{-2}.

Capillary electrophoresis–mass spectrometry

Although a detailed description of capillary electrophoresis (CE) is not within the scope of this paper, it is necessary to describe the basic elements of the CE experiment in order to appreciate the problems associated with coupling the technique to mass spectrometry. CE is performed in lengths of flexible capillary tubing filled with an appropriate buffer solution, similar to the buffers used in conventional electrophoresis. A small volume of sample (typically $<2\%$ of the column volume) is introduced into the capillary, across which an electric potential (*ca.* 20–30 kV) is then applied. Charged species inside the capillary exhibit different electrophoretic mobilities, and are thereby separated. Other components of a CE system include a high-voltage power supply, buffer reservoirs and electrodes, an injection system and a suitable detector.

Unlike HPLC, CE does not involve a mobile phase *per se*, which may be used to sweep known volumes of analyte solution onto the column from an injection valve. There are, however, several injection techniques available in CE, including hydrostatic, hydrodynamic (pressure or vacuum), split-flow and electrokinetic methods. A brief description of the injection methods evaluated during this study will be given later. It should be noted that, except for the electrokinetic injection mode, the method of sample injection is also the means by which the capillary is filled with the running buffer. When using untreated fused-silica capillary columns, in order to maintain reproducible migration times and electrophoretic performance, it is necessary to flush the column with several column volumnes (*ca.* 10 μl) of the running buffer after each analysis. In addition, it is also common practice to wash the column regularly with a weak base (typically a 0.1 *M* solution of sodium hydroxide) to remove adsorbed material from the walls of the capillary. Most of the available commercial systems have motorized carousels allowing wash–fill–inject–run sequences to be programmed into a method file.

Detection in CE is usually achieved by spectrophotometric methods; an optical window is typically created in the column itself by burning off a portion of the polyimide coating of the fused-silica capillary. As mass spectrometry is a destructive technique, however, the exit of the CE column must be physically connected or *interfaced* to the ion source of the mass spectrometer. The methods used to achieve this are described below.

Both the liquid-junction and coaxial approaches to achieving CE–MS were evaluated. Both approaches utilized a modified commercial fully-articulated IonSpray interface and the different configurations are shown schematically in Fig. 1. The fully-articulated interface is a development of the original ISP probe and has been designed for ease of optimization and greater flexibility in the coupling of chromatographic and electrophoretic systems. The commercial interface consists of two zero dead volume tee-junctions mounted on an articulated arm attached to the clear plastic source flange by a system of vernier micrometers. The first tee is used for the introduction of the ISP nebulizing gas, and the second may be used as a post-column splitter in HPLC or for CE coupling (described below). The micrometers provide movement of the arm in the axial and both lateral directions (x, y, z), and allow precise positioning of the ISP needle relative to the sampling orifice. The arm itself is pivoted, allowing the ISP needle to be moved at an angle (ϕ) to the sampling orifice. This additional degree of freedom was included as it has been found that, under certain conditions, a significant reduction in background cluster ions can be obtained by sampling the spray at an oblique angle to the interface plate and sampling orifice.

Coaxial interface. Based on previous experience with a coaxial arrangement of fused-silica capillaries for the coupling of packed capillary columns with continuous-flow FAB-MS [28], a preliminary coaxial CE–MS interface was used to confirm peak identities in the recent development of a CE–UV method for PSP toxins [26]. The principal difference between this preliminary interface and the current design is that the length of the coaxial sheath capil-

lary is greatly reduced in the latter, thereby lowering the back-pressure generated so that an inexpensive syringe pump may be used to deliver the make-up flow. The design also makes it much easier to change the CE column. The zero dead volume tee nearest the orifice was replaced with one drilled-out to accommodate a larger ISP needle through which the sheath capillary, containing the CE column, was fed (Fig. 1).

The important parameters in the construction of this particular configuration are the tight fits of the various concentric capillaries inside one another and their relative positioning at the probe tip. The I.D. of the in-house manufactured metal nebulizer sheath was drilled to accommodate the ISP needle (20-gauge stainless-steel tubing) with a snug fit, as the interspacial gap provides the restriction to the nebulizer gas against which its flow is controlled. The ISP needle protrudes *ca.* 0.5 mm from the nebulizer sheath to provide a focused spray of charged droplets. In later designs we hope to dispense with the fused-silica sheath capillary by extending the metal ISP needle to the second tee. The exit of the CE column (360 μm O.D., 50 μm I.D.) is held 0.5 mm inside that of the ISP needle, and the sheath capillary (500 μm O.D., 390 μm I.D.) a further 0.5 mm back from the CE column. This arrangement ensures good mixing of the effluents from the sheath capillary and column, minimizes the dead volume and maintains good electrical contact between the combined effluent and the ISP needle.

The make-up buffer for the sheath capillary is delivered to the back tee by a syringe pump, via a submicroliter injector (60-nl loop), at 3–8 μl/min. Formic acid (0.2%) was found to be the most suitable make-up buffer for positive ion analyses. At higher concentrations, the increased conductivity led to high CE currents (tripping the safety interlock on the CE systems) and instability in the ISP ion current. The injector provides a rapid means of optimizing the interface by flow-injection analysis (FIA). Another advantage of this injector was that it was found to provide a means of independently determining the amount of material injected on the CE column by injecting a suitable standard after the CE–MS experiment.

Liquid-junction interface. In this configuration the back zero dead volume tee (Fig. 1) is replaced by a modified tee which has one side replaced with a glass window to allow accurate alignment of the CE column and a transfer line (140 μm O.D., 75 μm I.D.) attached to opposite sides of the tee. The distance between the two capillaries is critical to the performance of this configuration. If it is too large the analyte, on exiting the CE column, can diffuse into the junction, leading to a loss in electrophoretic performance, whereas if the capillaries are too close, insufficient make-up buffer may be drawn into the flow into the transfer line to maintain a stable ion current. Thus, a gap of *ca.* 10–20 μm was found to be optimum. The make-up reservoir (a 1-ml syringe) is mounted on the interface flange, 3–5 cm above the ISP needle. The make-up buffer enters the transfer capillary via a combination of gravity and the venturi vacuum generated by the escaping nebulizing gas at the end of the ISP needle. In operation, a flow-rate of 2–3 μl/min was measured by observing the loss from the reservoir over a period of time.

The two configurations are readily interchangeable; with experience it was found to be possible to change from one to the other in a few minutes, although obtaining the correct gap in the liquid-junction configuration often required several attempts.

RESULTS AND DISCUSSION

Evaluation of injection techniques for CE–MS

The coaxial CE–MS interface was used for all of these experiments, as it was possible to perform flow-injection analysis (FIA) via the make-up flow and thereby independently obtain quantification of electrophoretic peaks. While the hydrostatic (or siphon) injection technique, in which either the sample end of the capillary is raised or the detector end (*i.e.*, the mass spectrometer) is lowered, has been used successfully for CE–MS by other workers [20], in our hands the technique was found to be impractical, and was not used during this investigation. However, it was important to keep the ends of the capillary at the same level to provent siphoning. Similarly, it has not so far been possible to envisage a simple apparatus which applies a vacuum at the end of the capillary (similar to that used in the ABI system) once it was positioned within the CE–MS interface; unlike conventional mass spectrometers there is no vacuum in the API ion source which may be utilized for injection.

Pressure injection (Beckman). In this approach,

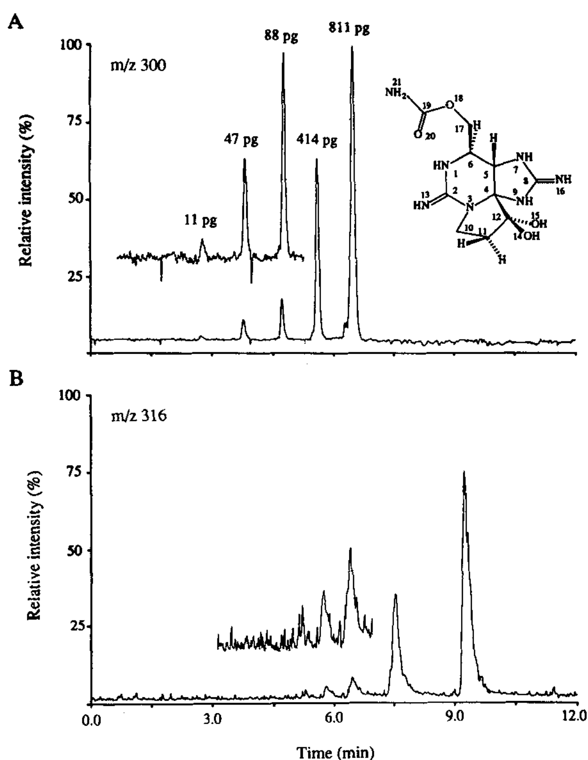

Fig. 2. Separation of the PSP toxins (A) saxitoxin and (B) neosaxitoxin by CE–MS using selected ion monitoring of MH^+ ions. Conditions; consecutive 10-s pressure injections of serial dilutions (1:1, 1:2, 1:10, 1:20 and 1:100) of a 500 μg/ml (each) solution, Trisma buffer (pH 7.2), voltage 24.4 kV (effective), coaxial CE–MS interface using make-up of 0.2% formic acid at 8 μl/min. Column, 90 cm × 50 μm I.D.

the sample end of the capillary and the electrode are inserted through gas-tight septa of individual sample and/or buffer vials. To inject, the sample vial is pressurized (*ca.* 0.5 p.s.i.) for a set time, forcing a small volume of the solution into the CE column. The amount injected is obviously dependent on the dimensions of the column, and on the viscosity of the solution. One of the criteria used to compare the injection methods was linearity of response, and Fig. 2 shows the CE–MS response obtained by consecutive 10-s pressure injections of various dilutions of a mixture of the PSP toxin saxitoxin (STX) and its N_1-hydroxylated analogue, neosaxitoxin (NEO), using selected ion monitoring (SIM) of their respective protonated molecules (MH^+). The electrophoretic process was stopped for each injection (made at 1-min intervals) by turning off the CE voltage, and it can be seen that the peak widths increase with the order of injection due to diffusion of the analytes while the electrophoresis was stopped and the next pressure injection performed. Comparison of the two ion electropherograms shows that each corresponding pair of the two toxins is well resolved, with neosaxitoxin migrating after saxitoxin. Peak-area measurements for saxitoxin were compared with those of standard solutions obtained by FIA following the electrophoresis experiment to give the amounts indicated in Fig. 2. From these amounts and the known concentrations it was estimated that *ca.* 1.8 nl was injected on to the CE column. It can be seen that the detection limit of saxitoxin is *ca.* 10 pg with a signal-to-noise ratio of 2. Even though this approach was used simply to illustrate the flexibility of the injection system and would not be the method used to generate a calibration graph, the CE–MS response for both toxins was found to be linear over two orders of magnitude (5–500 μg/ml).

Split-flow injection (ISCO). This method of injection is similar in principle to the split injection technique in gas chromatography (GC), in which only a portion of the analyte solution injected is introduced on to the column. An HPLC syringe (10 μl) is used to inject the sample into one port of a three-way injection block [29]. The tip of the CE column seats into the top port by means of a septum seal and the third (split-vent) port, opposite the syringe port, is plumbed with capillary tubing of varying lengths (3–5 cm) and/or I.D., (0.005–0.020 in.) which provides the splitting ratio.

The selected ion electropherogram (SIE) obtained from the CE–MS analysis of a mixture of five antibiotics used in the aquaculture industry using a 1-μl split-flow injection is shown in Fig. 3B. Only four peaks are observed as erythromycin (ETH) comigrates with ometoprim (OMP). The separation was performed at 24.4 kV (effective) using Trisma buffer (pH 7.2). One of the advantages of using the coaxial design with this injection technique is that the splitting ratio can be accurately calculated by comparison of the electrophoretic response with the FIA response obtained after the electrophoretic experiment is complete. Using a standard solution of trimethoprim (TMP), a split ratio of 1:60 was calculated for this analysis, indicating an injection volume of the order of 16 nl, which represents 116 pg on-column of each component (7.1 μg/ml) except

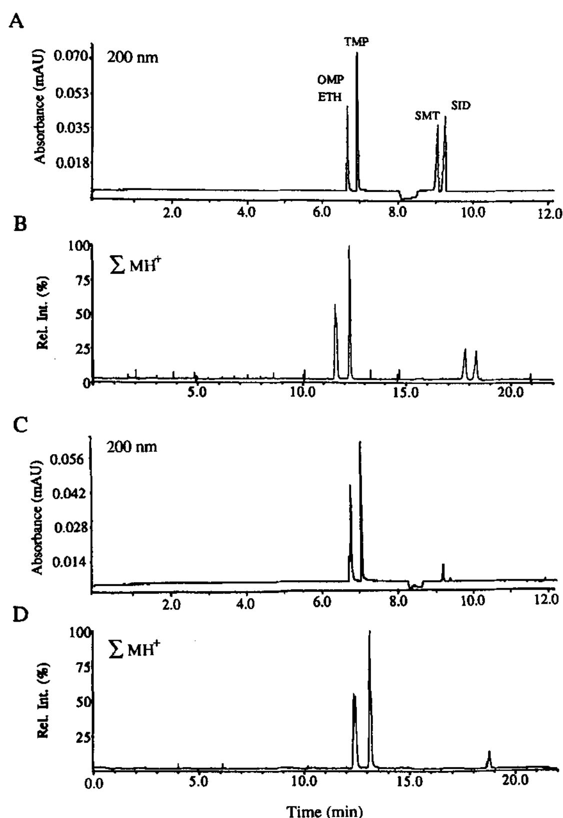

Fig. 3. Separation of antibacterial drugs used in the aquaculture industry by (A and C) CE–UV (200 nm) and (B and D) CE–MS (SIM). Conditions as in Fig. 2 except injection for (A) 3-s vacuum, (B) 1-μl split-flow (1:60) and (C and D) 10-s electrokinetic (5 kV).

erythromycin, which at 71.4 μg/ml represents 1.16 ng on-column.

The CE–MS SIE compares favorably with the CE–UV trace shown in Fig. 3A, which was obtained using vacuum injection (ABI) and 24 kV. The difference in migration times between the CE–UV electropherogram and the CE–MS SIE is due to the fact that UV detection occurs 22 cm before the end of the capillary. In order to compare the electrophoretic performance of the CE–MS interface with that obtained using the UV detector, the plate numbers were calculated for the single component peaks. The plate numbers (and peak width at half-height) for the trimethoprim peak by CE–UV and CE–MS were 225 000 (2.1 s) and 181 500 (4.1 s), respectively. Although the latter represents a 20% loss in electrophoretic efficiency over the CE–UV analyses, it should be recalled that mass spectral detection occurs 22 cm after the UV and diffusion of the analyte during migration over this additional length of capillary would account for a significant portion of the observed loss in efficiency. Unfortunately, the physical dimensions of the electropherograph and mass spectrometer are such that they could not be brought closer together, although a smaller CE system has been constructed in this laboratory, which will allow much shorter columns to be used. These and similar comparisons indicate that the coaxial interface can maintain both chromatographic integrity and the high separation efficiencies available with CE. These comparisons also confirm the effectiveness of the split-flow method for sample introduction in CE–MS. The quantitative performance of the split-flow injection method, however, was not so encouraging. Although reproducibility was obviously dependent on the accurate dispensing of 1-μl volumes, obtaining and maintaining the correct splitting ratio were found in practice to be the major problems associated with this injection technique. Using replicate injections it was found that peak areas could vary by as much as ± 10%. Partial blocking of the split-vent tubing by a build-up of salts was found to be a major source of irreproducibility.

Electrokinetic injection (all three CE systems). This mode of injection, in which the analyte migrates into the column under an applied field, is perhaps the easiest to accomplish in that no special vials, seals or plumbing of gas/vacuum lines are required. Typically, a voltage of 5 kV is applied for a fixed time (*ca.* 1–30 s) before the capillary is returned to the running buffer and the analysis started. Despite its simplicity, there is a problem due to electrophoretic discrimination associated with this injection mode, in that the amount of a particular analyte injected will be proportional to its electrophoretic mobility. This is illustrated in Fig. 3D, which shows the SIE of the mixture of five antibiotics using a 10-s electrokinetic injection (it should be noted that the ISP voltage is turned off during the electrokinetic injection). Comparison with the corresponding SIE in Fig. 3A reveals that the late-migrating isomeric sulfonamides, sulfamethazine (SMT) and sulfaisomidine (SID), both show extensive discrimination. The corresponding CE–UV trace obtained using an electrokinetic in-

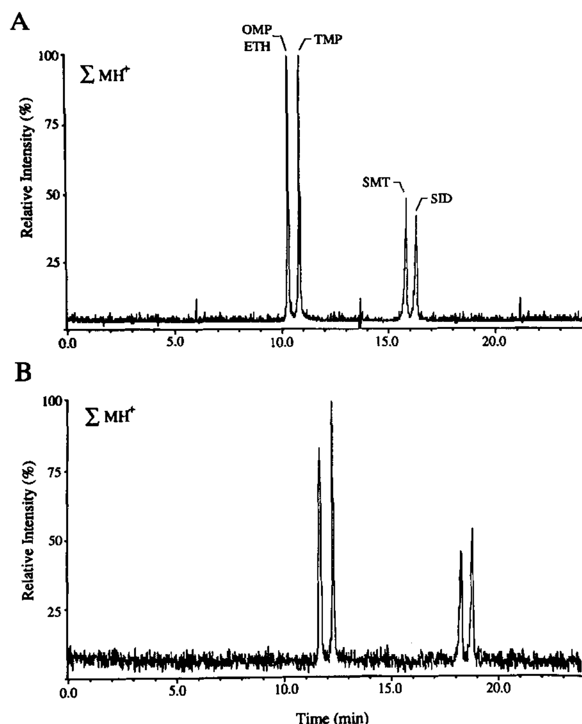

Fig. 4. Separation of antibacterial drugs by CE–MS using (A) coaxial and (B) liquid-junction interface configurations. Conditions as in Fig. 3B.

jection is presented in Fig. 3C, and confirms that the discrimination is not merely an artifact of the CE–MS interface. Indeed, the two UV electropherograms (Fig. 3A and C) were run consecutively on the same CE system. A second disadvantage of the electrokinetic injection method is that the amount of sample injected is also a function of the sample buffer composition. This can cause problems in the analysis of complex extracts containing high levels of salts.

Comparison of CE–MS interface configurations

The CE–MS SIEs of the five antibiotics, using the coaxial and liquid-junction configurations, are shown in Fig. 4A and B, respectively. The analyses were performed on the same day, under indentical CE conditions using a 1-μl split-flow injection. The most obvious difference between the two CE–MS separations is the migration time. Although the analyses were conducted on the same column, the migration times are longer in the case of the liquid junction. These differences were initially believed to be due simply to the additional length of transfer capillary (8 cm) used in the latter configuration. However, the 2 μl/min flow-rate through the transfer capillary (as determined by the observed loss of make-up buffer from the reservoir with time) is higher than that through the column, and thus the residence time is at most 30 s and does not account for the 1–1.5 min difference in migration times. In considering a possible explanation for the observed differences, a potential disadvantage of the liquid junction was revealed. It was noted that the current recorded with the liquid junction was *ca.* 10–12 μA higher than that during the coaxial analysis, and this was believed to result from a difference in the buffer composition at the interface. When filling and washing the capillary between runs with the liquid-junction, it was found that the effluent does not exit into the ion source, as in the coaxial configuration, but takes the path of least resistance and flows into the make-up reservoir line. To minimize this problem, the column was re-filled and washed with minimum volumes of buffer solutions (2–5 μl), and the interface was left for 10 min prior to injection in order for sufficient make-up buffer to wash through the junction. The possible influence of this changing composition on the sensitivity of the ISP process is discussed below.

Closer examination of the individual traces reveals a further loss of electrophoretic performance with the liquid-junction interface, in addition to that already described in the comparison between the CE–UV and CE–MS (coaxial) electropherograms (Fig. 3). The calculated plate numbers for trimethoprim, with the coaxial and liquid-junction configurations, were 169 800 and 101 500, respectively. This represents a 1.67-fold difference for the two interfaces. Similar improvements in calculated plate numbers were found for the other antibiotics with the coaxial relative to the liquid-junction configuration: ometoprim (2.29), erythromycin (1.21), sulfamethazine (2.43) and sulfaisomidine (3.59). Consideration of the expected additional band broadening with the inclusion of the liquid-junction transfer capillary does not account for all of the observed loss in plate number, and this is attributed to the junction itself. This is supported by other workers [25] who, using fluorescence detection, reported a tenfold decrease in plate number with the inclusion of a similar liquid-junction.

The other notable difference is the higher noise level in the liquid-junction SIE (which actually comes predominantly from only one ion, *m/z* 279). This is believed to be due, in part, to the lower flow-rate of make-up buffer (0.2% formic acid), which results in an increased contribution of the CE buffer (Trisma) to the background. Peak-area measurements showed that the responses for ometoprim, trimethoprim, sulfamethazine and sulfaisomidine were 47, 44, 49 and 31% higher, respectively, with the coaxial than with the liquid-junction arrangement, although interestingly the response for the higher molecular weight erythromycin was 28% higher with the latter. It is possible that the splitting ratio is different in the two configurations, with the liquid-junction generating a higher back-pressure

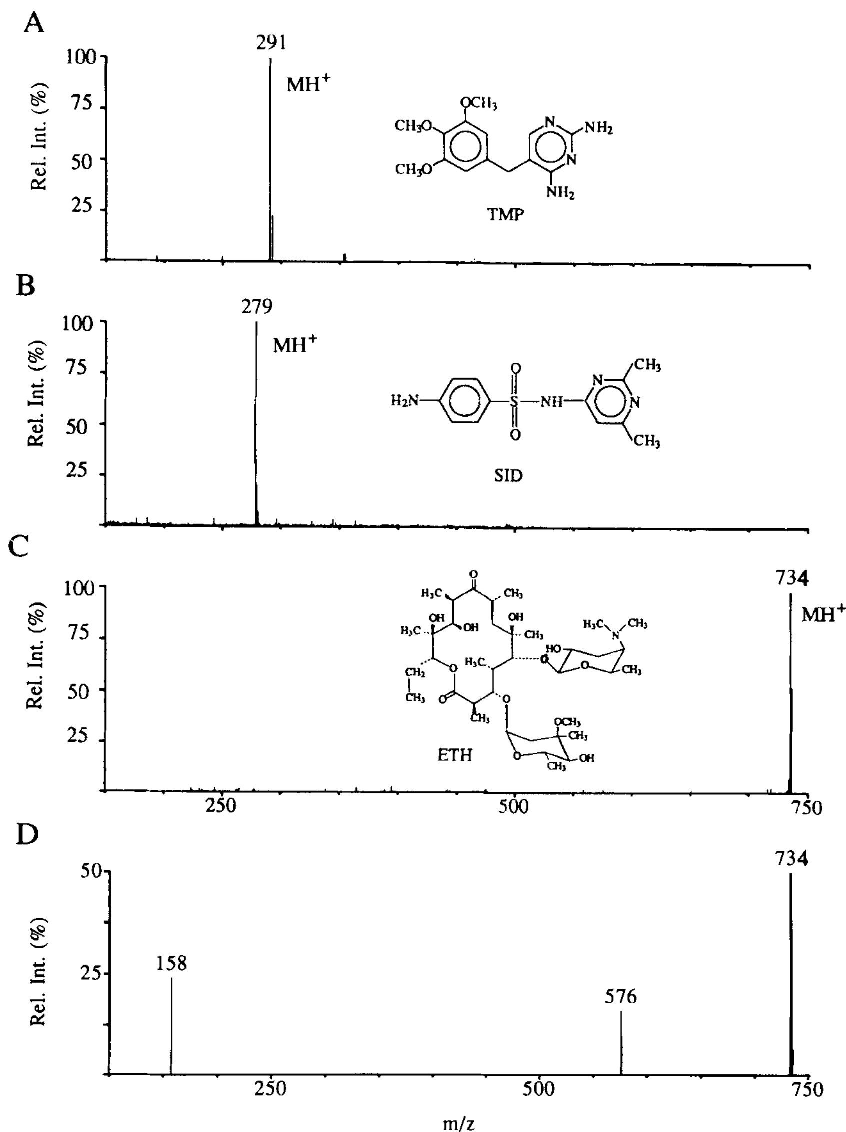

Fig. 5. Background-subtracted CE mass spectra of (A) trimethoprim (TMP), (B) sulfaisomidine (SID) and (C) erythromycin (ETH). CE conditions as in Fig. 3. Mass range, 150–750 dalton. (D) MS–MS of erythromycin by flow-injection analysis. Collision energy (laboratory frame), 35 eV; target gas (argon) thickness, $3.5 \cdot 10^{14}$ cm^{-2}.

than the coaxial configuration, resulting in less sample being injected in the former interface. This is thought to be unlikely as the make-up flow to the junction is through wide-bore Teflon tubing. It is also possible that the changing composition of the make-up buffer in the liquid junction may affect the ISP response. Without the ability to use FIA with this interface it is not possible to substantiate these arguments.

Full-scan analyses were also performed with this mixture of antibiotics, and the full-scan (150–750 dalton) CE–MS mass spectra of trimethoprim, sulfaisomidine and erythromycin are shown in Fig. 5A, B and C, respectively. These spectra are typical of those obtained by the mild ISP process, consisting of simply the protonated molecule and little or no fragmentation. The spectra of ometoprim, trimethoprim and sulfaisomidine are identical with those recently obtained by LC–MS [14]. Full-scan CE–MS detection limits for the antibiotics were not as good as expected from the excellent SIM results. It is pertinent at this time to mention a limitation of scanning mass spectrometers as detectors for CE. As discussed above, the high-efficiency separations currently achieved in CE–MS result in peak widths of the order of 3–5 s. The problem is a more extreme version of the well known dilemma in capillary GC–MS, where the chromatographic peak widths require a mass spectrometer scan cycle of no more than *ca.* 1 s in order to avoid serious distortions of the mass spectra. In addition, sample loadings are smaller in CE, so that the question of the signal-to-noise ratio in the mass spectra is even more pressing than in GC–MS. The volume of analyte solution can be no more than *ca.* 1% of the column volume if optimum CE performance is to be maintained; this implies a maximum injection volume of *ca.* 20 nl, a factor of 50–100 smaller than the typical injection volumes in capillary GC–MS. Therefore, a compromise must be found for individual CE–MS experiments, in which mass spectral quality (signal-to-noise ratio and freedom from distortion) is traded off against mass scan-range and speed, and also against CE peak width via injection volume. Based on experience with a range of analytes with molecular weights in the range of a few hundred daltons, *ca.* 1 ng of sample injected on-column is required if a mass spectrum of adequate signal-to-noise ratio is to be acquired at a scan rate of *ca.* 250 dalton/s

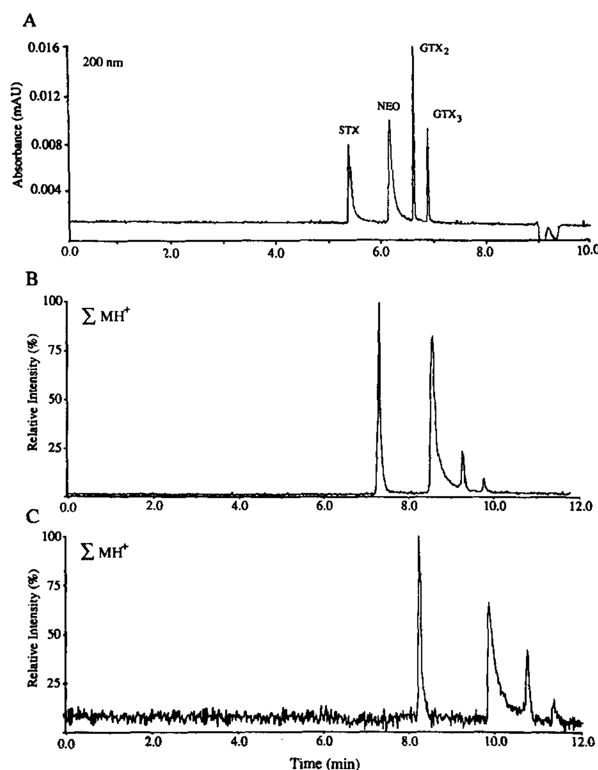

Fig. 6. Separation of PSP toxins isolated from a culture of the dinoflagette spp. *Alexandrium excavatum* by (A) CE–UV (200 nm) and by CE–MS using (B) coaxial and (C) liquid-junction interface configurations. Conditions as in Fig. 3B.

using ISP CE–MS. These constraints can be removed if a non-scanning mass spectrometer is used, either a focal-plane instrument with a channel electron multiplier array detector [30] or an ion trap permitting ion storage over the duration of the CE peak.

The electrophoretic separation of saxitoxin, neosaxitoxin and the C_{11}-sulfated gonyautoxins (GTX-2 and GTX-3), performed at 24 kV using UV detection at 200 nm, is presented in Fig. 6A. The toxins, isolated from a strain of *Alexandrium excavatum* dinoflagellates, are well resolved although the neosaxitoxin peak shows significant tailing owing to interaction of the hydroxyl group with the capillary walls. The corresponding CE–MS separation using the coaxial and liquid-junction interfaces are shown in Fig. 6B and C, respectively. The SIEs consist of the respective MH^+ ions (*m/z* 300, 316 and 396) and compare favorably with the UV trace,

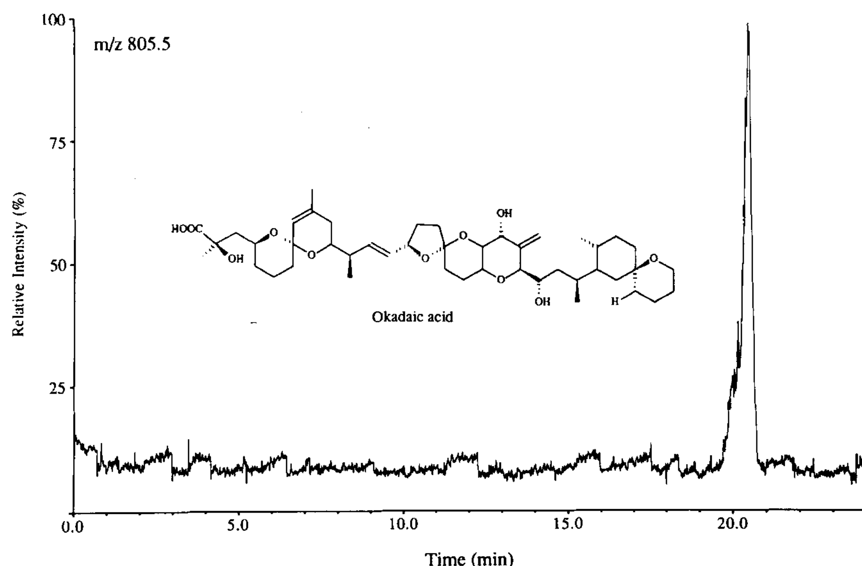

Fig. 7. CE–MS SIM response for 16 ng of the DSP toxin okadaic acid (m/z 805.5). Conditions as in Fig. 4A.

providing unambiguous confirmation of UV peak identities. The relative abundances of the peaks corresponding to the gonyautoxins were found to be higher in the CE–UV electropherogram. This is due to the fragmentation of the labile sulfated analogues under ISP ionization [26], via loss of the sulfate (80 dalton) to give the charged species $[M+H-SO_3]^+$ at m/z 316, which coincidently is the same mass as the protonated molecule of neosaxitoxin. The differences between the two SIEs are similar to those discussed above, with the migration times being slightly longer with the liquid-junction interface.

CE–MS was also applied to other marine toxins of concern to the Canadian aquaculture industry. Fig. 7 shows the SIE obtained from the CE–MS analysis of okadaic acid, the principal toxin associated with DSP [4], using the coaxial interface. Under the electrophoretic conditions used (Trisma, pH 7.2), the lipid-soluble polyether toxin migrates in its anionic form, as evidenced by the characteristic (fronting) electrophoretic profile. Quantification of the peak by FIA after the electrophoretic experiment indicated 16 ng of the toxin was injected on-column. In this case, significant improvements in sensitivity will have to be made in order for CE–MS to offer an alternative to the ISP LC–MS method for DSP toxins recently described [15]. The application of the CE to the analysis of domoic acid, the neurotoxin responsible for amnesic shellfish poisoning [3], has been recently reported [31]. Fig. 8A shows the reconstructed ion electropherogram of the MH^+ (m/z 312) from the full-scan (150–350 dalton) CE–MS analysis (CAPS, pH 11) of the instrument calibration solution of the toxin (DACS-1, 80 μg/ml) available from this laboratory under the Marine Analytical Chemistry Standards Program. The CE mass spectrum taken at the top of the electrophoretic peak is presented in Fig. 8B, and resulted from the introduction of 1.6 ng of the toxin.

Capillary electrophoresis with tandem mass spectrometry (CE–MS–MS)

The use of MS–MS with ISP-MS to provide structural information on sulfonamides and their potentiators has been described recently [14]. The product ion spectra of protonated trimetoprim, ometoprim, and the isomeric sulfonamides, obtained by CE–MS–MS, were identical with those obtained previously by LC–MS–MS [14]. The CID product ion spectrum of protonated erythromycin obtained by FIA (via the coaxial make-up buffer inlet) is shown in Fig. 5D, and compares favorably with that reported during a recent investigation of

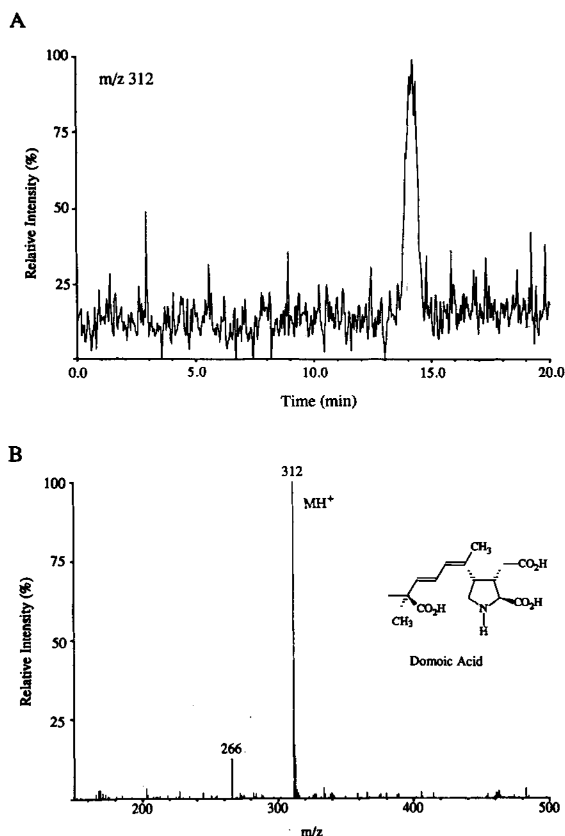

Fig. 8. Full-scan analysis of the neurotoxin domoic acid by CE–MS. (A) Reconstructed ion electropherogram of MH^+ (*m/z* 312) and (B) background-subtracted mass spectrum. Conditions: CAPS buffer (pH 11), voltage 24.6 kV (effective), mass range 150–500 dalton.

the MS–MS of erythromycin and other macrolide antibiotics using FAB ionization on a tandem sector instrument [32]. Two major product ions are observed at *m/z* 576 and 158. These ions are indicative of bond cleavage accompanied by hydrogen rearrangement at the site of the glycosidic oxygen. As a result, the non-amino sugar is lost as a neutral fragment (156 dalton) to give the product ion at *m/z* 576, but the amino sugar is observed as a charged species (*m/z* 158).

Product ions of antibiotics thus identified by MS–MS may be used in CE–MS–MS methodologies, either to provide increased selectivity by using selected reaction monotoring (SRM) or to aid in the identification of related species by using precursor scans of common product ions. The former technique is illustrated in Fig. 9, which shows the SRM electropherograms obtained from CE–MS–MS analysis of the mixture of the five antibiotics under conditions identical with those described above, monitoring the dissociation of MH^+ ions to characteristic product ions. For the isomeric sulfonamides two reactions were monitored, *viz*, *m/z* 279 > 156 (Fig. 9C) and *m/z* 279 > 213 (Fig. 9B). The latter ion is assigned to the loss of (H_2, SO_2), and it can be seen that sulfamethazine selectively shows this fragmentation, thus providing both electrophoretic and mass spectrometric confirmation of this isomer.

The application of CE–MS and CE–MS–MS to the analysis of a shellfish extract fortified with sulfadimethoxine (SDM) at the 1 ppm level is demonstrated in Fig. 10A and B, respectively. The CE–MS SIM electropherogram of the MH^+ ion of sulfadimethoxine (*m/z* 311) shows a peak close to the migration time observed for the standard and well re-

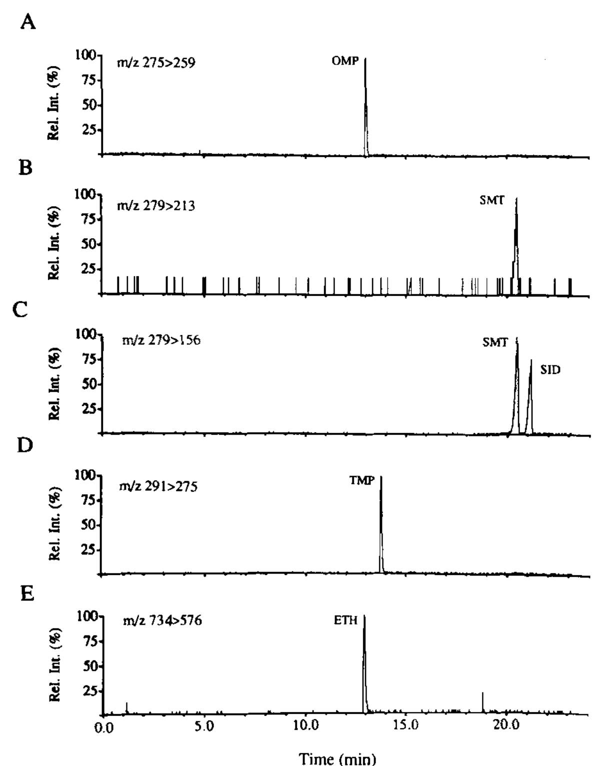

Fig. 9. Separation of antibacterials by CE–MS–MS using selected reaction monitoring. CE conditions as in Fig. 4A and MS–MS conditions as in Fig. 5.

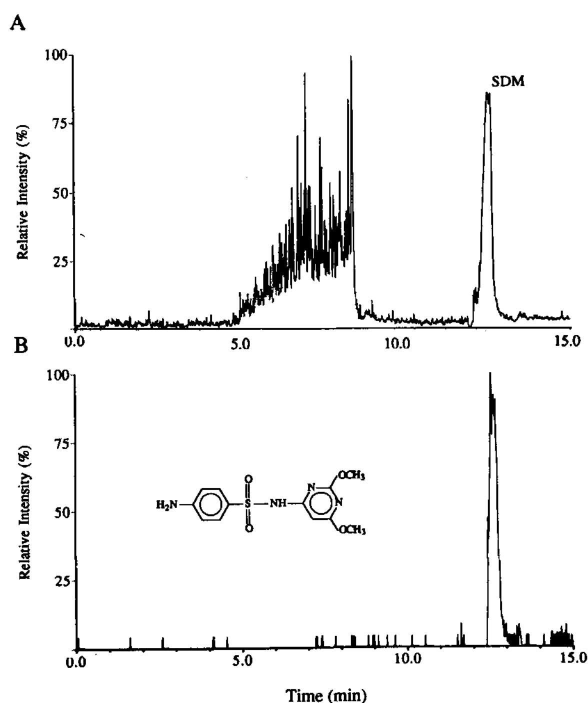

Fig. 10. Analysis of shellfish extract fortified with sulfadimethoxine (1 ppm) by (A) CE–MS using SIM (*m/z* 311) and (B) CE–MS–MS using SRM (*m/z* 311 > 156). CE conditions as in Fig. 4A and MS–MS conditions as in Fig. 5.

solved from a significant degree of interference in the early part of the electropherogram. The observed CE–MS peak width for sulfadimethoxine is significantly larger than those observed for standard sulfonamides, and is assigned to the effect of high salt concentrations in the extract. The corresponding CE–MS–MS SRM electropherogram (Fig. 10B), monitoring the dissociation of the MH^+ ion to *m/z* 156 (a common product ion of the sulfonamides [14]), demonstrates the increased selectivity obtained via the significant reduction in chemical noise.

CONCLUSIONS

Although both interface designs have been shown to be capable of providing efficient coupling of CE to API-MS, it is felt that the coaxial configuration provides a more robust and reproducible interface. The major advantage of the coaxial over the liquid-junction interface is that the make-up flow is delivered independently of the CE column effluent. This provides greater flexibility in the type of CE buffer that may be used, and offers the potential for FIA and even LC–MS of other samples via the make-up capillary without dismantling the CE interface. The use of FIA also provides an independent means of quantification, and this can also be used to determine accurately the splitting ratio when using a split-flow injection system. One other advantage of the coaxial configuration was more recently realized during the analysis of high-molecular-weight proteins by CE–MS. The FIA of a calibrant just prior to a CE–MS analysis permits mass accuracies, similar to those obtained by infusion, to be achieved. The application of these CE–MS interfaces to the analysis of proteins will be reported separately.

All three CE instruments performed well in the present CE–MS experiments. The only feature which differentiated significantly amongst them in this regard was the technique used for sample injection. Of the different injection modes evaluated for CE–MS, pressure injection seems to offer the best means of introducing the sample and associated buffer solutions into the CE column, without disturbing the interface. Compared with the electrokinetic injection technique, pressure injection shows no discrimination amongst analytes on the basis of electrophoretic mobility.

It has been shown that CE–MS and CE–MS–MS can be used to analyze a variety of compounds of concern to the aquaculture industry. There are some additional concerns, however. One drawback of CE is that migration times and separation efficiency are dependent on the composition of the analyte solution, and for regulatory work the use of suitable migration markers and internal standards would be a necessity. Another general problem arises as a consequence of the low sample volumes (1–30 nl) which can be injected without degradation of electrophoretic separation. The implications of this restraint can be appreciated most easily by considering an contaminant with a regulatory limit of 1 μg per gram of fish tissue (1 ppm). The extract from 1 g of tissue would most likely be made up to a total volume of 1 ml, in order to keep the total concentration of co-extracted salts and other endogenous material down to manageable levels. A 10–20-nl injection of such a solution contains 10–20 pg of the

target analyte. A useful analytical technique should be capable of operating below the regulatory limit by at least a factor of ten, *i.e.*, for CE methods the detection limit must be of the order of 1–2 pg (for a 1 ppm limit). This detection limit may be improved by either improved clean-up procedures, preconcentration of the sample or larger injection volumes, although the last two options are both undertaken at the cost of degradation in electrophoretic efficiency.

Nevertheless, using a combination of selected ion and selected reaction monitoring, CE–MS is currently capable of confirming the presence of antibiotics at the low ppm level in complex matrices. Improvements in the CE analysis of real extracts, which should bring the CE–MS detection limits for these compounds below the regulatory limit (0.1 ppm), and in line with those available by ISP LC–MS [15], are currently under investigation.

With regard to the marine toxins examined, the results obtained for the PSP toxins are particularly encouraging. Considering the challenge that these labile toxins present to the analytical chemist, CE offers a real alternative to the HPLC–fluorescence detection method currently used for the regulatory monitoring of shellfish, with regard to both sensitivity and ease of use. Full details of the application of CE–MS for the analysis of PSP toxins in biological extracts will be reported elsewhere [33]. The CE–MS instrumental detection limit for okadaic acid is sufficiently high that it seems highly unlikely that this technique will ever form the basis of a routine analytical method for underivatized DSP toxins. The case of domoic acid is slighly more promising, but it is felt that at present the CE methodology cannot successfully compete with LC–MS.

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