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Original Paper

Quantification of domoic acid in shellfish tissues by pressurized capillary electrochromatography

A method was developed to quantify domoic acid (DA), the chemical responsible for amnesic shellfish poisoning (ASP), by pressurized CEC (pCEC). The effect of different experimental conditions on the separation of DA and matrix solutes, such as the content of ACN in mobile phase, pH and concentration of buffer, supplementary pressure and applied voltage, were investigated. Under the optimal conditions, the pCEC method separated DA from shellfish matrices within 6 min. By using supplementary pressure, bubble formation in the capillary column was completely suppressed. The method was repeatable, sufficient accurate and sensitive for rapid screening of DA in shell seafood.

Keywords: Amnesic shellfish poisoning / Pressurized capillary electrochromatography / Risk assessment / Seafood

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1 Introduction

The syndrome known as amnesic shellfish poisoning (ASP), including abdominal cramps, vomiting, disorientation and memory loss [1], has been ascribed to consumption of shellfish containing domoic acid (DA). Most countries have set a regulatory limit of 20 μg DA/g wet weight (ww) of shellfish tissues to protect consumers [2].

Up to now, numerous methods have been developed for the analysis of DA. The mouse bioassay developed by the Association of Official Analytical Communities (AOAC) to test for paralytic shellfish poisoning (PSP) toxins has also been used to detect DA [3], but this classical approach has significant drawbacks such as poor reproducibility, low sensitivity, interference from matrices, and so on [4]. Rapid assay approaches using biochemical techniques can be used to screen samples for the presence of DA if such methods have a small rate of false negatives [5]. Chemical methods, especially those that involve separation prior to detection, are still accurate and sensitive analytical methods for regulatory purposes to quantify this toxin [6]. HPLC with UV detection is the method that has been most widely used for quantifying DA in shellfish, and this method has been validated for mussels by AOAC [7]. Methods using HPLC separation with MS or fluorescence detection have also been developed for detecting DA in samples of shellfish, phytoplankton, and seawater [6]. While, these methods are sensitive and acceptable, methods employing CE [8–10], and CEC [11, 12] have an important advantage over HPLC methods: relative small sample consumption. This advantage can be very attractive in the marine toxins field since routine monitoring is often hindered due to small mounts of sample and standards available for marine toxins [13]. Furthermore, CE and CEC can provide faster and simpler determination of the marine toxins. These advantages make both CE and CEC attractive alternatives to HPLC.

The preliminary results on application of CEC to analysis of DA [11] suggested that the main limitation of CEC was poor reproducibility due to bubble formation, which made the method insufficiently robust [12]. Pressurized CEC (pCEC) uses an HPLC pump coupled to the inlet end of the capillary column to provide supplementary pressure [14]. The practical problems associated with bubble formation and column drying that occur in CEC can be overcome by use of a pCEC system [15]. Also, applying supplemental pump pressure increases flow-rate and shortens separation time [16]. Finally, quantitative sample introduction in pCEC can be achieved through a rotary-type injector [17]. These characteristics of pCEC provide better separation selectivity and repeatability than CEC. Based on the previously published CEC method by Leão Martins *et al*. [11], here we describe the development and application of the pCEC-UV method for the rapid quantification of DA in shellfish tissue extracts.

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Abbreviations: pCEC, pressurized CEC; **DA**, domoic acid; **WW**, wet weight

2 Materials and methods

2.1 Materials

A packed capillary column (100 μm/id × 375 μm/od, total length 55 cm, of which 20 cm was packed with 3 μm octadecyl silica (ODS) particles) was obtained from Global Chromatography (Su Zhou, China). The DA standard (≥97.0%, HPLC-grade) was purchased from Sigma–Aldrich (USA). HPLC-grade ACN and methanol were obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China). The other chemicals used were of analytical grade. High purity deionized water was prepared with a Milli-Q water purification system (Milford, MA, USA).

2.2 Apparatus

pCEC was performed on a commercially available Trisep™ 2100GV pCEC system (Unimicro Technologies, Pleasanton, CA, USA) equipped with a UV/Vis detector (190–600 nm). The system was comprised of a microvolume pump, a high-voltage power supply (0 ~ ±30 kV), a microfluid manipulation module (including a six-port injector), and a chromatogram acquisition unit configured as previously described [18]. Samples were injected in the external 2 μL sample loop and then carried by the mobile phase to the four-port split valve. The total mobile phase flow from the four-port valve was split into two paths, one entered the capillary column under constant pressure controlled by a back-pressure regulator, and another went to the waste reservoir. A negative voltage was applied to the outlet of the column, and the inlet of the column was connected to the split valve and grounded. Data collection was performed using a chromatographic workstation system (Qianpu Software, China).

2.3 Sample preparation

Samples of mussel were purchased at a local market. The method initially proposed by Quilliam [5] with slight modification was used for extraction. Four grams of homogenized mussel tissue were added to 16.0 mL of methanol/water (1:1 v/v) then homogenized for 3 min and then centrifuged for 10 min at 3600 rpm. The supernatant was filtered through a dry methanol-compatible 0.22 μm filter and kept at 4°C until analysis. To optimize separation parameters, mussel tissue extract spiked with 5 mg/L DA was used.

2.4 pCEC procedures

Compounds were separated by isocratic elution and detected by UV absorption at a wavelength of 242 nm. The mobile phase was prepared by mixing an appropriate volume of ACN and Tris buffer solution, and degassed in an ultrasonic bath for 20 min. A supplementary pressure (7.2 MPa) was applied to the column inlet and the flow rate of the pump was set to 0.050 mL/min. Before conducting the pCEC experiments, the column was conditioned with mobile phase for 1 h and the temperature of the column was kept at room temperature (25°C). The column was equilibrated for 30 min after the mobile phase was changed. The relative humidity in the laboratory was kept less than 70%. The applied voltage was first ramped from 0 to −13 kV and then maintained at −13 kV.

3 Results and discussion

3.1 Optimization of the separation and determination conditions

3.1.1 Effect of composition of the mobile phase

Three types of buffer solution, phosphate, borate, and Tris, were tested as buffers in the mobile phase containing 60% v/v of ACN. In the Tris buffer, the components of the mussel tissue extract such as tryptophan and some endogenous compounds [19] were eluted together and fewer electrochromatographic peaks than in borate and phosphate buffer were obtained. This resulted in a better separation of DA from components in the matrix. Furthermore, Tris buffer has a lower conductivity, such that a higher voltage could be used without causing great background current and Joule heating in the capillary column. Therefore, the Tris buffer was chosen as the composition of mobile phase.

In order to determine potential effects of organic modifiers on separation by pCEC, the ACN concentration was varied from 50 to 70% v/v in the mobile phase while keeping the Tris buffer concentration at 5 mmol/L (pH = 8.0) and an applied voltage of −11 kV (Fig. 1). Retention time of DA was inversely proportional to ACN concentration. The lower ACN concentration would benefit the separation, but longer analysis time was needed. A concentration of 60% v/v ACN, which gave the best compromise between speed of analysis and separation selectivity, was chosen for further experiments.

3.1.2 Effect of concentration and pH of the buffer

The effect of Tris concentration on separation of DA and matrix solutes was studied by using 5, 25, and 50 mmol/L of Tris (pH = 8.0). Retention times of DA and matrix solutes were increased with greater concentrations of Tris, whereas resolution kept unchanged. Increasing the Tris concentration lead to greater background current which caused Joule heating, so 5 mmol/L of Tris was selected as the buffer in the mobile phase.

DA possesses three carboxyl groups and one amino group with pK_a values of 2.10, 3.72, 4.97, and 9.82 respec-

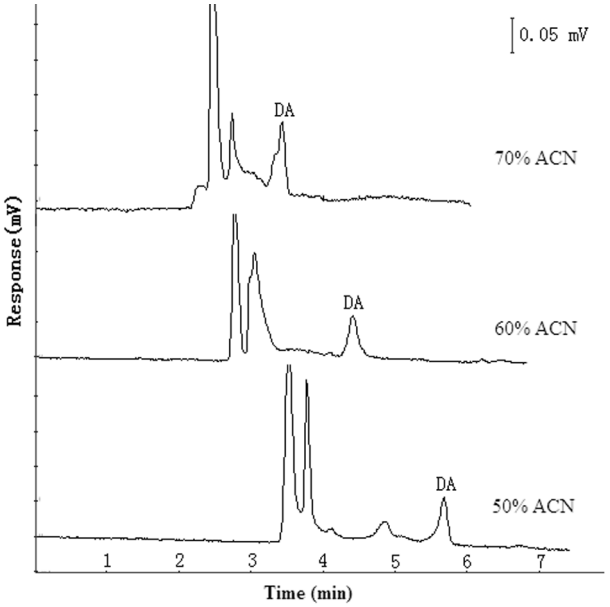

Figure 1. Effect of volume fraction of ACN on separation of mussel tissue extract spiked with 5 mg/L DA. Experimental conditions, packed capillary column (100 μm/id × 375 μm/od, total length 55 cm, of which 20 cm was packed with 3 μm ODS particles); mobile phase, ACN-5 mmol/L Tris buffer solution (pH = 8.0), the volume fraction of ACN as shown in this figure; applied voltage, –11 kV; supplementary pressure, 7.2 MPa; flow rate, 0.050 mL/min; detection wavelength, 242 nm. Peaks before DA are of matrix solutes.

tively. Therefore, depending on different pH values, DA can exist in a range of charged states (−3 to 1) [1], and can be analyzed by CE in either cationic or anionic mode with either acidic or basic buffers, respectively [8]. At pH values from 4.0 to 8.0, the effect of pH on migration behavior was investigated in anionic mode with a mobile phase consisting of 60% v/v ACN and 5 mmol/L Tris buffer solution. The best separation selectivity and speed of analysis were achieved at a pH of 8.0, where components of the sample matrix such as tryptophan form positive ions migrating toward the cathode and DA forms a negative ion migrating toward the anode. These opposite electrophoretic directions would benefit separation. Simultaneously, higher pH values generate relatively strong EOF which benefits shortening analysis time. For all of these considerations, a pH of 8.0 was chosen as the optimum for analyses of DA.

3.1.3 Effect of supplementary pressure

Although capillary packed columns have been widely used in CEC, the presence of discontinuities in a packed column in the form of the inlet and the outlet frits can cause bubble formation [20], which is a main problem associated with CEC [12]. In the pCEC method, this problem can be overcome by the introduction of supplementary pressure. Meanwhile, the overall velocity of the mobile phase was increased with the introduction of pressurized flow besides EOF. So analyses by pCEC could be conducted more rapidly and more reproducibly than CEC [16]. Varying from 3.6 to 7.2 MPa, the effect of supplementary pressure applied to the column inlet on separation was studied. Analysis time decreased from 15 to 6 min along with the increase in supplementary pressure due to an increase in linear velocity of the mobile phase. Because supplementary pressure was applied, there were no bubble peaks observed during any of the pCEC separations. The supplementary pressure of 7.2 MPa was selected for shortest analysis time.

3.1.4 Effect of applied voltage

The effect of applied voltage on the separation was examined over the range of −7 to −17 kV at 7.2 MPa supplementary pressure (Fig. 2). The retention time of matrix solutes decreased when the applied voltage increased. Migration time of DA increased as applied voltage increased from −7 to −13 kV, whereas when the applied voltage increased from −13 to −17 kV, migration time of DA decreased. In pCEC, the mobile phase is driven by EOF combined with pressurized flow, and the linear velocity of a charged solute in the mobile phase (u_m) is the sum of the velocities contributed from the electrophoretic velocity (u_{ep}), pressurized flow (u_p), and EOF (u_{eo}) (Eq. 1) [21].

$$u_m = u_{eo} + u_{ep} + u_p \quad (1)$$

The pressurized flow kept unchanged under constant supplementary pressure. The EOF and electrophoretic velocity increased as voltage increased from −7 to −17 kV. Considering that DA forms a negative ion at pH 8.0 migrating to the anode in the opposite direction as the EOF, the electrophoretic velocity of DA must be faster than the EOF in the range of −7 to −13 kV, which could explain why the migration time of DA increased in this range. But in the range of −13 to −17 kV, the EOF increased nonlinearly with applied voltage, which may be caused by Joule heating produced in the column, and the EOF may be greater than electrophoretic velocity at this range, so the migration time of DA decreased along with the increase in voltage from −13 to −17 kV. Finally, an applied voltage of −13 kV was chosen for greatest resolution between DA and adjacent matrix solutes.

3.1.5 Effect of flow rate of pump

When voltage and pressure were held constant, decreasing the flow rate of pump from 0.150 to 0.050 mL/min resulted in a linear increase in the peak area of DA at the same concentration. When the pump flow rate decreased under constant pressure and voltage, the flow rate in the capillary column was constant, but waste flow rate decreased correspondingly. So the splitting ratio between the column flow rate and the waste flow rate

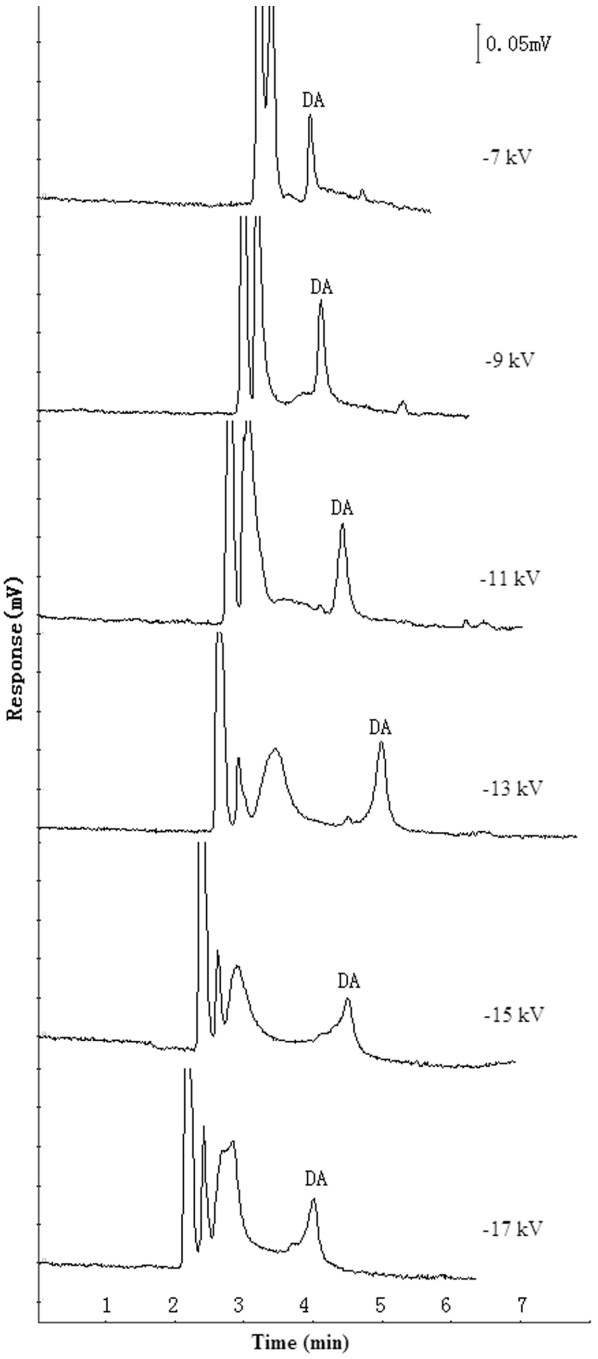

Figure 2. Effect of applied voltage on separation of mussel tissue extract spiked with 5 mg/L DA. Experimental conditions, mobile phase, 60% v/v ACN, 40% v/v of 5 mmol/L Tris buffer solution (pH = 8.0); supplementary pressure, 7.2 MPa; applied voltage as shown in this figure. Other conditions are the same as in Fig. 1.

increased. The injected sample volume is controlled by the splitting ratio since the pCEC instrument is equipped with an external loop with a fixed volume and a four-port split valve [18]. Along with the increase in splitting ratio, both the injection sample volume and signal intensity increased. As a result, a flow rate of 0.050 mL/min was selected to maximize signal intensity.

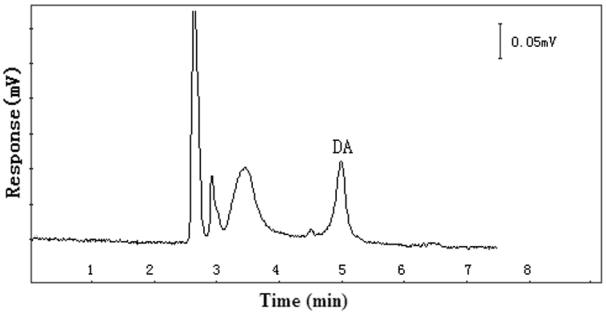

Figure 3. Typical electrochromatogram of mussel tissue extract spiked with 5 mg/L DA under the optimal conditions. Peaks before DA are of matrix solutes.

3.2 Final optimization

To sum up, the optimal conditions for separation of DA with matrix solutes were as follows: mobile phase, 60% v/v ACN, 40% v/v 5 mmol/L Tris buffer (pH 8.0); applied voltage, −13 kV; supplementary pressure, 7.2 MPa; flow rate, 0.050 mL/min. Figure 3 is a typical electrochromatogram of DA with matrix solutes under the optimal pCEC conditions.

3.3 Interferences by amino acids

Since the extract of the biological matrix contains a number of amino acids [19], acromatic amino acids, which can be directly detected by UV spectrophotometry, were selected to study their interferences on the determination of DA. Tryptophan, tyrosine, and phenylalanine were all eluted before DA under optimized conditions, and had good resolution with DA. Thus these compounds would not interfere with quantification of DA by pCEC.

3.4 Analytical performance

3.4.1 Calibration curve, LOQ, LOD, and repeatability

DA was quantified by external standard calibration. A series of concentrations of DA ranging from 0.5 to 100.0 μg/mL were tested to determine the calibration parameters under the optimized conditions. The linear range, regression equation, correlation coefficient, LOQ, and LOD are listed (Table 1). The calibration curve of DA was linear in the range of 1.0–100.0 μg/mL with $r = 0.9994$. The instrumental LOD was 0.5 μg/mL (S/N = 3) (equivalent to 2.0 μg DA/g, ww of mussel tissue, which was the method LOD). The method LOD is less than the guideline value in mussels (20 μg DA/g, ww of mussel tis-

Table 1. Parameters for quantification of DA

Compound	Regression equation[a)]	Correlation coefficient (r)	Linear range (μg/mL)	LOQ (μg/mL)[b)]	LOD (μg/mL)[c)]
DA	$y = 135.5x - 51.377$	0.9994	1.0–100.0	1.0	0.5

a) y, peak area (mV × s); x, amount concentration (μg/mL).
b) Based on the lowest concentration where the RSD (%) is estimated to be <5%.
c) Based on the S/N = 3.

Table 2. Retention time and peak area repeatability

DA	RSD for time (%)	RSD for area (%)
Run-to-run (n = 7)	0.49	2.08
Day-to-day (n = 7)	1.58	3.01

Table 3. Mean recovery of DA from mussel sample spiked at three concentrations of DA (n = 6)

Added (μg/g)	Recovery (%)	RSD (%)
5.0	83.4	3.0
10.0	85.2	2.3
20.0	86.4	2.8

sue). The sensitivity of this technique is sufficient to quantify DA in the naturally contaminated samples for regulatory purposes. The repeatability of retention time and peak area exhibited excellent precision of this established pCEC method (Table 2).

3.4.2 Spike recovery

Mean recoveries of DA that had been spiked into mussel tissue at concentrations of 5.0, 10.0, and 20.0 μg DA/g ww of mussel tissue were between 83 and 86% (Table 3).

3.4.3 Analysis of other shellfish samples

Samples of shellfish including oysters, razor clams, and scallops were purchased at a local market and analyzed using this established pCEC method. The obtained results showed components of extraction of these three shellfish were nearly the same as that of mussels and had no influence on the determination of DA, suggesting that the pCEC method developed here could be used in a wide range of shellfish matrices.

4 Concluding remarks

This work provides a rapid and simple method for the determination of DA in shellfish tissues by pCEC. Under the optimized conditions, DA can be baseline separated from shellfish matrices within 6 min. By using a pCEC system, the primary problem of bubble formation in reported CEC method was solved, and good repeatability with RSD less than 5% were obtained. Compared with the authorized HPLC method and reported CE methods, pCEC is a powerful alternative method for monitoring DA in shell seafood and holds promise for application in routine screening of DA in natural extracts.

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The authors declared no conflict of interest

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