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Short communication

Automatic high-performance liquid chromatographic method for the determination of diarrhetic shellfish poison

C. Hummert*, J.L. Shen, B. Luckas

Institute of Nutrition and Environment, Friedrich-Schiller-University of Jena, Dornburgerstrasse 25, D-07743 Jena, Germany

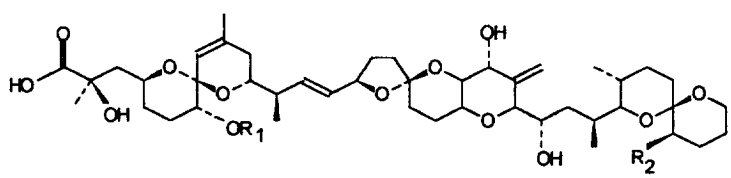
Abstract

A rapid HPLC method with fluorescence detection for the determination of okadaic acid (OA) and dinophysistoxin-1 (DTX-1) in mussels and mussel products is presented. For fluorescence labeling of OA and DTX-1, 9-anthryldiazomethane (ADAM) is used. HPLC with a column-switching system is proposed to avoid time-consuming clean-up procedures after derivatization of sample extracts with ADAM. The column-switching system as well as the chromatographic conditions and detection are described.

Keywords: Shellfish; Column switching; Okadaic acid; Dinophysistoxin-1; Phycotoxins; Toxins

1. Introduction

After consumption of shellfish having been fed with toxic dinoflagellates of the *Dinophysis* or *Prorocentrum* genera, intestinal disturbances such as diarrhoea, nausea, vomiting, abdominal pain and chills have been observed [1,2]. Since in most cases diarrhoea was the predominating symptom, the syndrome is called 'diarrhetic shellfish poisoning (DSP)'. Okadaic acid (OA) and dinophysistoxin-1 (DTX-1) (see Fig. 1) are the toxins responsible for the diarrhetic symptoms [3–6]. Therefore, it became necessary to establish methods for the evaluation of possible hazards caused by contamination of seafood with these phycotoxins. HPLC methods for the de-



Okadasäure (OA) : $R_1 = H$ $R_2 = H$
Dinophysistoxin-1 (DTX-1) : $R_1 = H$ $R_2 = CH_3$

Fig. 1. *Dinophysis* toxins.

* Corresponding author.

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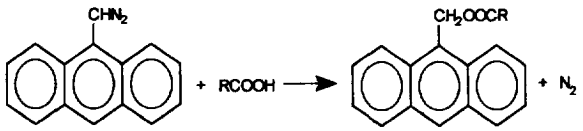

Fig. 2. Reaction of 9-anthryldiazomethane (ADAM) with organic acids.

termination of the DSP toxins are characterized by pre-column derivatization with 9-anthryldiazomethane (ADAM) (see Fig. 2) [7], followed by chromatographic separation of the 9-anthrylmethyl esters (9-AM-OA and/or 9-AM-DTX-1) and fluorescence detection [8–11].

However, the application of the reaction with ADAM requires an additional clean-up step after derivatization using silica cartridges. The clean-up procedure is time-consuming and is associated with losses of 9-AM-OA and 9-AM-DTX-1, respectively [12]. To avoid this time-consuming procedure with negative effects on the recovery, the HPLC equipment was modified by incorporating a column-switching system, which allows injection of the ADAM-derivatives directly into the chromatographic system [13].

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a Gilson M 305 master pump and Gilson M 306 piston pump, Gilson 806 manometric module, Gilson 234 autoinjector, Gilson 715 HPLC system controller software (Gilson Medical Electronics, USA); RF-10A spectrofluorometric detector (Shimadzu Corporation, Japan); Rheodyne switching valves, electric powered (Besta-Technik GmbH, Germany).

2.2. Chemicals

Okadaic acid (no. 495604) and *Dinophysistoxin*-1 (no. 317480) were obtained from Calbiochem-Novabiochem International (USA); acetonitrile (ROTI®SOLV HPLC no. 7330.2) and methanol (ROTI®SOLV HPLC no. 73421) were supplied by Carl Roth (Germany); dichloromethane ("pro analysi", no. 1.06050), water (Lichrosolv no. 1.15333) and acetone ("pro analysi", no. 299.0500) were from E. Merck (Germany); 9-anthryldiazomethane (research grade, no. 13682), was from Serva Feinbiochemica (Germany).

2.3. Sample extraction

A 1.0-g amount of sample and 4.0 ml methanol–water (80:20, v/v) were homogenized with an ultra-turrax for 3 min and centrifugated for 5 min at 2980 *g*. A 2.5-ml volume of the supernatant was transferred to a separatory funnel and extracted twice with 4.0 ml dichlormethane. The dichlormethane phases were filled up to 10.0 ml in a graduated test tube.

2.4. Derivatization

A 1.0-ml volume of the dichlormethane extract was carefully evaporated to dryness under nitrogen and 200 μl of ADAM solution (0.15% in acetone) were added to the residue. Derivatization was carried out at 25° in darkness for 90 min. An aliquot (20 μl) was directly injected onto the HPLC system with column-switching system.

2.5. Chromatographic conditions

Column A: RP-C-8, 250 × 4.6 mm I.D., Supelcosil LC-8-DB 5 μm (no. 58354, Supelco, Germany); column B: RP-C18, 100 × 4 mm I.D., MN-Cart Nucleosil-C-18, 5 μm (no. 721215, Macherey-Nagel, Germany); column C: RP-C-18, 250 × 4.6 mm I.D., Supelcosil LC-18, 5 μm (no. 58298, Supelco, Germany); flow-rate A (pump A), 1.6 ml/min; flow-rate B (pump B), 1.1 ml/min; eluent A, acetonitrile–water (65:35, v:v); eluent B, acetonitrile–water (90:10, v:v); detection, fluorescence (ex: 365 nm, em: 415 nm); injection, 20 μl; Cut window: for 9-AM-OA, 15.0 min–18.0 min and for 9-AM-DTX-1, 29.0–32.0 min.

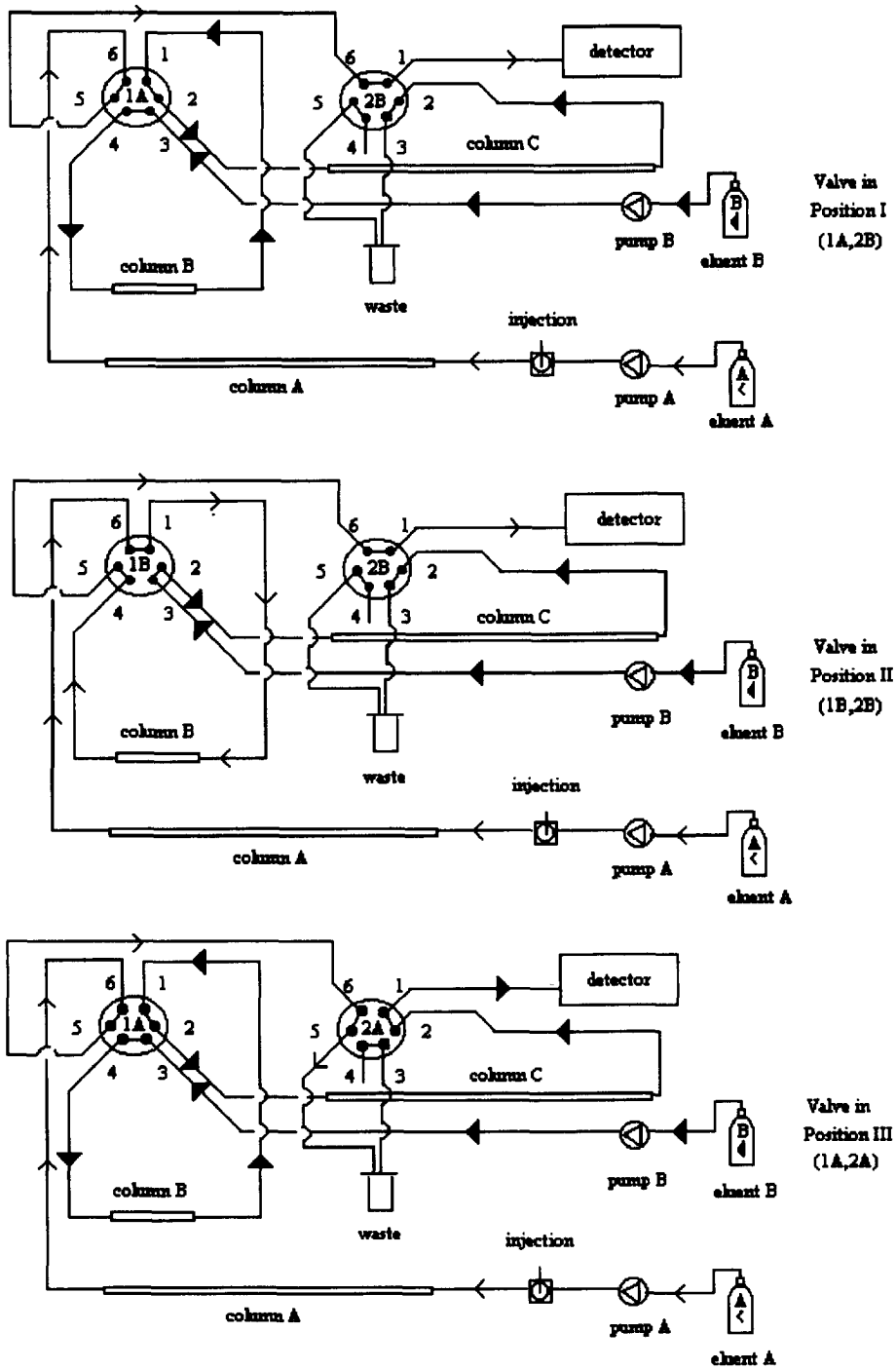

Fig. 3. Column-switching system for the HPLC determination of DSP-toxins in mussels and mussel products.

2.6. HPLC with column-switching system

A C_8 column (column A: 250 × 4.6 mm I.D.) and two C_{18} -columns [trap column B (100 × 4.6 mm I.D.) and column C (250 × 4.6 mm I.D.)] were combined with two switching valves, named '1' and '2', both with two possible positions, named 'A' and 'B' (see Fig. 3). After injection of 20 μl of the derivatized sample the analysis starts with the valves in position I (valve 1 = A, valve 2 = B). In this position the sample is pre-separated on column A, while column B and C are rinsed by pump B. The detector is connected to column A.

Just before 9-AM-OA or 9-AM-DTX-1 are leaving column A the valves are switched to position II (valve 1 = B, valve 2 = B). The 9-AM esters eluting from column A are fixed on column B.

After the total amount of the 9-AM esters has been trapped on column B the valves are switched to position III (valve 1 = A, valve 2 = A). The derivatized DSP-toxins are eluted from column B to column C and they are detected after separation at the fluorescence detector which is now connected to column C. Column A is rinsed by pump A in valve position III.

To analyse sample extracts the retention times of 9-AM-OA and 9-AM-DTX-1 have to be exactly ascertained. To find out the moments when the valves have to be switched, derivatized standard solutions with high amounts of DSP-toxins are injected onto the HPLC system in

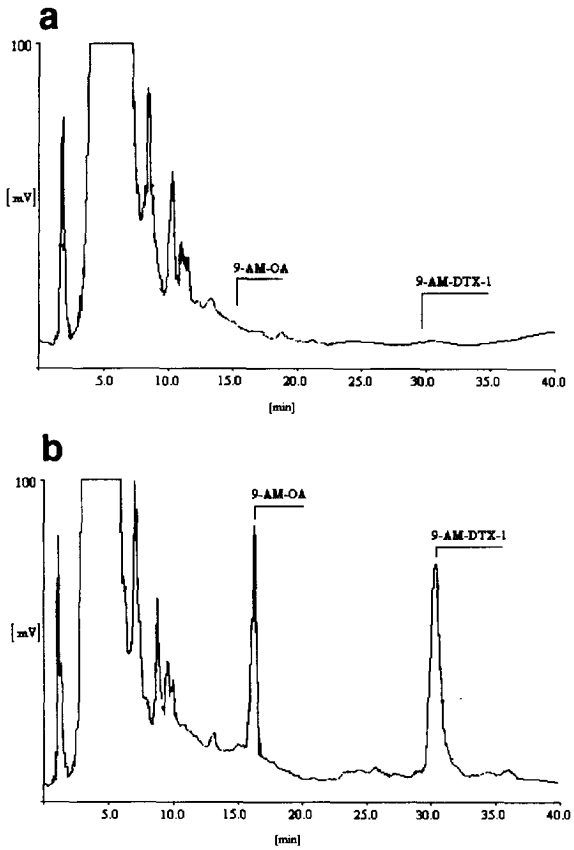

Fig. 4. (a) Chromatogram of an ADAM-derivatization solution (blank) (only C_8 column A without any cut). (b) Chromatogram obtained from a DSP standard solution (50 ng OA, 75 ng DTX-1), derivatized with ADAM (only C_8 column A without any cut).

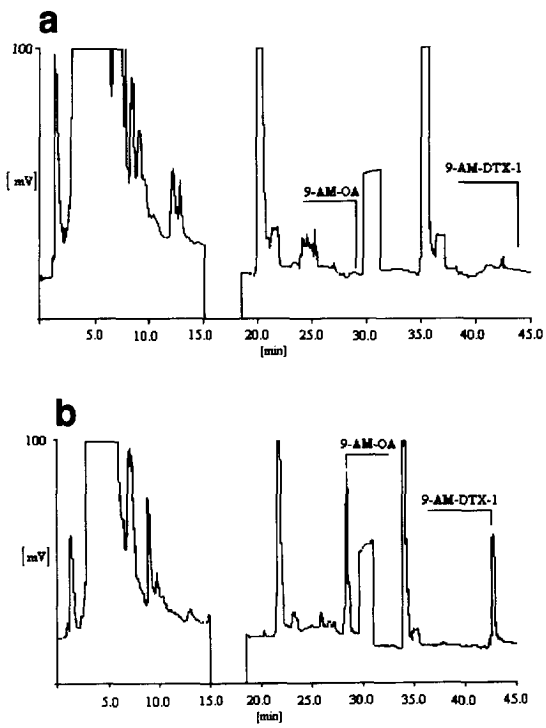

Fig. 5. (a) Chromatogram obtained from an uncontaminated mussel extract (blank), derivatized with ADAM (cut 1, 15.0–18.0 min; cut 2, 29.0–32.0 min). (b) Chromatogram obtained from a DSP standard solution (10 ng OA, 10 ng DTX-1), derivatized with ADAM (cut 1, 15.0–18.0 min; cut 2, 29.0–32.0 min).

valve position 1 and analysed without any switching. Fig. 4 shows chromatograms obtained in this manner.

3. Results

Figs. 5 and 6 present chromatograms obtained with the HPLC method with column-switching system. Fig. 5a shows no interfering peaks at the retention time of 9-AM-OA and 9-AM-DTX-1 caused by the derivatization reagent ADAM or matrix components while Fig. 5b demonstrates that OA and DTX-1 are quite well detectable. The detection limit for both OA and DTX-1 as the 9-AM-esters is 0.5 ng (at a signal-to-noise ratio of 5) [12]. By application of the presented sample preparation and derivatization procedure, a toxin content of 0.08 mg DSP/kg sample material is detectable. The allowed amount of DSP-toxins in Europe is 0.4 mg/kg sample material [13]. Examples of low DSP contaminated sample materials are shown in Fig. 6a and Fig. 6b. The chromatograms in Fig. 6 demonstrate that 2 ng OA (i.e. 0.32 mg DSP/kg) are rapidly and unambiguously detectable with sufficient sensitivity.

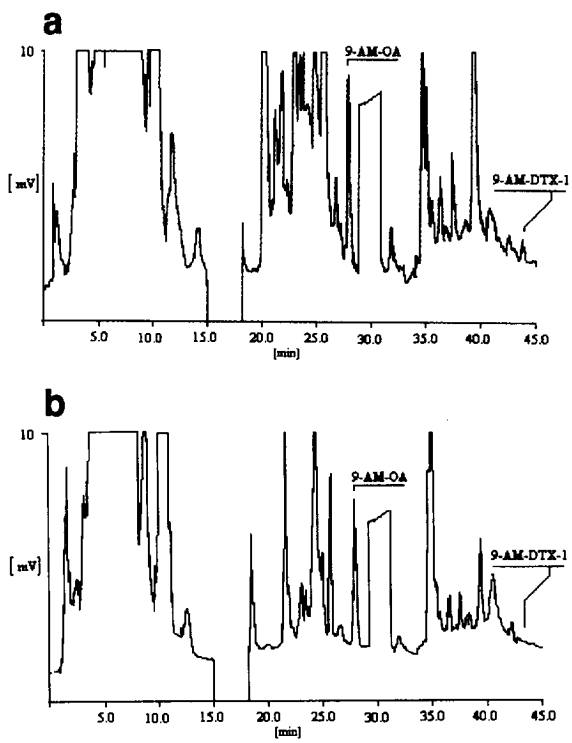

Fig. 6. (a) Chromatogram obtained from a contaminated hepatopankreas extract (Limfjord, North Sea), contaminated with OA (2.0 ng) and DTX-1 (traces), derivatized with ADAM (cut 1, 15.0–18.0 min; cut 2, 29.0–32.0 min). (b) Chromatogram obtained from a contaminated mussel extract (Sylt, North Sea), contaminated with OA (2.0 ng), derivatized with ADAM (cut 1, 15.0–18.0 min; cut 2, 29.0–32.0 min).

4. Discussion

The application of HPLC with column-switching system permits a significant shortening of the time-consuming clean-up procedure. The recoveries of 9-AM-OA and 9-AM-DTX-1 were higher than 90%. Using the method with a silicagel clean-up procedure of the 9-AM-esters we could not achieve recovery rates higher than 40% [12]. The obtained chromatograms are free of interfering peaks from matrix and derivatization reagent. Thus, a rapid and selective HPLC method for the determination of DSP-toxins in mussels and mussel products is available. Using a computer with HPLC-manager and laboratory data system it is possible to work with electric switching valves to run the method automatically.

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