Journal of Chromatography B, 876 (2008) 245–251

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of domoic acid in mussels by HPLC with post-column derivatization using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and fluorescence detection

Marios Maroulis[b], Ioannis Monemvasios[a], Elisabeth Vardaka[a], Pantelis Rigas[a,*]

[a] *Biotoxins and Analytical Chemistry Laboratory, Department of Fisheries and Aquaculture Technology, Technological Educational Institute of Thessaloniki, N. Moudania, Greece*
[b] *Analytical Chemistry Laboratory, Chemistry Department, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece*

ARTICLE INFO

Article history:
Received 25 May 2008
Accepted 30 October 2008
Available online 5 November 2008

Keywords:
Domoic acid
Kainic acid
Shellfish poisoning
Biotoxins
Post-column detection
NBD-Cl
HPLC post-column fluorescence detection

ABSTRACT

A new, sensitive method was developed for the determination of the neurotoxin domoic acid (DA) using a reversed phase separation followed by post-column derivatization (PCD) with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and subsequent fluorescence detection. The PCD conditions which involves a two-step reaction was fully optimized for the lowest detection limit. The first reaction occurs between DA and NBD-Cl while the second makes possible the detection of the derivative causing the destruction of the interfering fluorescent 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) which is the hydrolysis product of NBD-Cl. Kainic acid a similar base structure compound with DA was used as an internal standard. The developed post-column method provides the ability for a fully automated analysis, low detection limits (LOD 25 ppb in real samples of mussel extracts), it requires less sample preparation, and it gives clean simple chromatograms without chromatographic interferences from coeluting compounds such as tryptophan. The method was successfully applied to for the quantitative determination of DA in mussel tissues at quantities as low as 75 μg/kg tissue.

1. Introduction

In 1987, 153 people suffered from intoxication after consuming blue mussels (*Mytilus edulis*) from Prince Edward inland and it was shown that the poisoning was caused by the neurotoxin, domic acid (DA) [1,2]. Since then DA has been detected in razor clams and crabs [3] and is being monitored in shellfish, mainly mussels, clams and oysters [4–6]. DA, which is produced by algae Pseudo-nitzschia [7,8] is ingested by Phytoplankton Shellfish and accumulates in their edible tissue. Consuming contaminated shellfish causes Amnesic shellfish poisoning to mammals, which in turn can cause permanent or short term memory loss as well as other neurological symptoms, such as severe headaches, loss of balance like nausea, vision disturbances, disorientation, etc. [9,10]. DA accumulates in small fish that feed on the algae.

In the European Union as well as Canada and the USA the legal limit for DA is 20 μg DA/g in edible tissues. High performance liquid chromatography (HPLC) with photodiode array detection (LC-UV) is the most popular method used for the determination of DA [11]. Bioassays can also be used for higher concentrations of DA but are lacking in sensitivity and selectivity with up to 20% variation in results.

One of the most common LC methods for the determination of DA in shellfish tissue is the one developed by Quilliam et al. in 1989 [1] using a reversed phase column, under isocratic elution, and an optical absorbance detection at 242 nm. For the trace determination of DA in seawater and phytoplankton (where DA levels are lower than the shellfish) the method developed by Pocklington et al. in 1991 with pre-column derivatization with fluorenylmethoxycarbonyl chloride (FMOC-Cl) and fluorescence detection following a gradient reversed phase separation, is often used [12]. This method can achieve a very low detection limit (15 ppb) but cannot be used for the analysis of DA in shellfish tissues due to interferences from compounds present in the shellfish matrix. One such strong interferent is the, tryptophan which elutes close to DA. A procedure using precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was developed and applied to the determination of DA in phytoplankton at 1 ppb [13]. A precolumn derivatization method based on a 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was shown to be suitable for the determination of DA

* Corresponding author. Tel.: +30 2373026457; fax: +30 2373026450.
E-mail address: rigas@aqua.teithe.gr (P. Rigas).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.053

[14]. Unfortunately, matrix co-eluted compounds are present in the chromatograms that might interfere with DA peak and the NBD-F is commercially available at very high cost and consequently limits its use in post-column applications. LC coupled to mass spectrometry (MS) detection has also been used for the determination of DA [15]. Although the LC-MS method is sensitive and selective, the overall methodology is complex and costly.

4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) were reported previously to be useful post-column reagents for the fluorometric detection of amino and imino acids after their separation by ion-exchange chromatography [16–18]. This work is focused on the development of a procedure for the LC reversed phase separation of DA followed by post-column derivatization (PCD) using NBD-Cl with subsequent fluorescent detection. PCD methods typically require less sample preparation and clean–up than precolumn derivatization methods, have less interferences from the reagents employed, and the results are overall more reproducible. Furthermore, NBD-Cl is commercially available at modest cost compared to that of the NBD-F. All parameters affecting the reversed phase separation of DA and those that affect the post-column derivatization of DA with NBD-Cl were identified and optimized in order to obtain the most favorable detection limits for the DA. When applied to mussel matrix samples and using kainic acid as an internal standard it was possible to determine DA at a detection limit of 25 ppb.

2. Experimental

2.1. Reagents

Domoic acid (DA) a certified calibration solution (CRM-DA-e) at a concentration of 99.4 μg DA/L in 1:9 acetonitrile:water solution and CRM-ASP-Mus-b certified reference shellfish material (36 μg DA/g) were obtained from National Research Council, Nova Scotia, Halifax, Canada. Water was produced by a SG Water Ultra Clear Water purification system. HPLC grade acetonitrile and methanol and kainic acid (>99%) were obtained from Sigma–Aldrich, St. Louis, MO, USA Ethyl acetate p.a. was purchased from Fluka, St. Louis, MO, USA. HCl (37% solution) and trifluoroacetic acid PB were obtained from Panreac, Barcelona, Spain, while 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was purchased from Fluka, St. Louis, MO, USA. All reagents were used as received.

2.2. Equipment

The LC system (Shimadzu Corporation, Kyoto, Japan) is composed of a Model LC-10ADvp solvent delivery module with FCV-10AL quaternary valve and DGU-14A degasser, a model SIL-10ADvp autosampler, a CTO-10ACvp column oven, a SCL-10Avp controller, a SPD-M10A VP Diode array detector, and a RF-10Axl fluorescence detector. Class-VP chromatography software was used for data collection/handling. The LC column was a Nucleosil C18, 250 mm × 4.6 mm, 5 μm, 100 Å column obtained by Macherey Nagel, Dueren, Germany. The post-column derivatization system (PCD) used was the model Prometheus PCD 300 Plus manufactured by Rigas Labs, Thessaloniki, Greece and included two heated reaction coils, as well as two reagent delivery Marathon I pumps. For sample preparation an IKA (Staufen, Germany) Ultra Turrax T25 blender, an Eppendorf (Hamburg, Germany) 5810R Centrifuge, and an Alltech Vacuum Manifold equipped with Alltech (Deerfield, IL, USA) solid phase extraction cartridges (500 mg, 3 mL SAX) and 0.45 μm nylon membranes were used.

2.3. Sample preparation

The procedure for handling mussel tissue was based on that published by Quilliam et al. in 1995 [19,20] and was followed with minor modifications. DA was extracted from the mussel tissue by homogenization with methanol-water (1:1, v/v). Approximately 50 g of mussel tissue was homogenized with the blender. A portion of this homogenized tissue was weighed directly on a precision balance. A 4.0-g homogenated tissue was extracted with 15.0 mL extraction solvent (1:1 methanol–water) and homogenized again for 10 min at 6500 rpm. The centrifuged sample was then filtered through a 0.45-μm membrane and was purified further using the following SPE procedure.

2.3.1. Cartridge conditioning

Initially 6 mL of methanol passed through the cartridge, followed by 3 mL water, and finally 3 mL extraction solvent (1:1 methanol–water) through the SAX cartridges.

2.3.2. Cartridge cleanup/elution

A 5.0-mL fraction of the filtered crude extract was loaded onto the cartridge in a flowrate of about one drop per second. The flow was stopped when the fluid's meniscus reached the top of the cartridge packing. Then the effluent was discarded. The cartridge was washed at about one drop per second with 5 mL wash solution (1:9 acetonitrile–water). Again the flow was stopped when the fluid's meniscus reached the top of the cartridge packing. Then the effluent was discarded and 0.5 mL of formic buffer eluent (0.5 M, pH 3.2 ± 0.2, adjusted with sodium hydroxide) were added and allowed to flow until the fluid's meniscus reached the top of the cartridge packing. Then the effluent was discarded again. A 2-mL volumetric tube was placed under the cartridge and 2 mL formic buffer were loaded onto the cartridge and allowed to flow at a rate of one drop per second. Exactly 2 mL of the final eluted DA extract were collected and then stored for up to 1 day.

An alternative method without SPE treatment is based using a 4.0-g homogenated tissue, extracted with 15.0 mL solvent (5:1 methanol–water) and homogenized again for 10 min at 6500 rpm. The centrifuged sample was preconcentrated under nitrogen to a final volume of 2 mL. This solution was then filtered through a 0.45-μm membrane and then injected to HPLC without any further clean-up steps.

2.4. Liquid chromatography – post-column derivatization

2.4.1. LC separation of DA

The mobile phase conditions and reversed phase column for the separation of DA were 0.1% trifluoroacetic acid (TFA) in 87:13 water:acetonitrile with a flow rate of 0.7 mL/min and Nucleosil C18 column, respectively. The composition of TFA and the percent acetonitrile in the eluent are the parameters affecting mostly the separation of the analytes (DA and Kainic acid (IS) a compound with similar base structure with DA) that for this reason they were fully optimized in order to obtain the best separation from the sample. Detection of the analytes was achieved with a fluorescence detector using a post-column derivatization reaction with two reagents. The detector was set at 469 nm λ_{ex} and 529 nm λ_{em} (the λ_{ex} and λ_{em} are the optimum excitation and maximum emission wavelengths for the derivative between the NBD-Cl and DA) with gain at 4×. Data were collected and processed with the Shimadzu Class-VP chromatography software.

2.4.2. Post-column derivatization conditions

The PCD system reagent 1 was optimized to be 9.0 mM NBD-Cl in methanol with the borate buffer adjusted to pH 10.0. The output

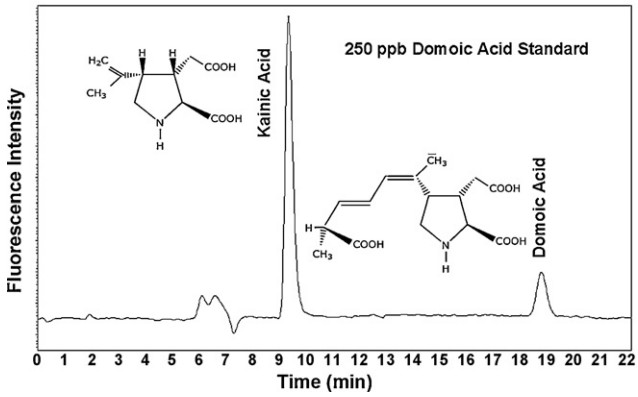

Fig. 1. Standard mixture of 250 ppb domoic acid (DA) with kainic acid (IS) using 17:83 0.1% TFA in H_2O:Acetonitile as mobile phase.

of the column and reagent 1 were mixed in a mixing tee cartridge of 50 μL volume and the mixed fluid was then passed through a 1.5-mL polyetheretherketone (PEEK) coil with internal diameter of 0.010 in. heated at 90 °C. A second reagent, reagent 2, containing 1 M HCl in ethyl acetate was mixed in another 50 μL mixing tee cartridge and passed through a 100-μL reaction PEEK coil before the eluent reached the fluorescent detector. A back-pressure regulator was adjusted to 100 psi in order to prevent the formation of gas bubbles in the fluorescence detector flow cell to minimize baseline noise. The variables affecting the post-column detection are: concentration of the two reagents, the pH, the buffer concentration, the type and composition of organic solvent, the coil volume and i.d. of the tubing, the temperature of the reagent coils, the flow rate of reagents and finally the cartridge's mixing volume.

3. Results and discussions

3.1. Chromatography and sample preparation optimization

The mobile phase conditions eluted the analytes isocratically with 87% H_2O with 0.1% trifluoroacetic acid (TFA) and 13% CH_3CN. Kainic acid is a compound with similar in structure with DA and thus it was used as an internal standard (IS) in the method. The concentration of this internal standard in all injected solutions was at 250 ppb level. The 0.1% TFA was found to be adequate to maintain the DA in its undissociated form using its reverse phase properties to provide substantial retention of it in the Nucleosil C18 column. The optimum concentration of acetonitrile was found to be between 15% and 20%, at which levels it provides excellent resolution between the analyte DA and IS thus there was no need to employ gradient elution. Initially at the beginning of the optimization process of the post-column conditions the mobile phase composed of CH_3CN:H_2O 20:80 were shown to be adequate (see Fig. 6) even though the ratio changed slightly to 17:83 for the analysis of the real samples in order to further increase the resolution (see Fig. 1). At the reduced acetonitrile concentration the matrix does not interfere at all with the IS peak.

Regarding the sample preparation optimization the SPE procedure using SAX cartridges was used initially in our studies taking into consideration two main factors. First for extended column life and second for the development of a robust method. However, this procedure is indeed unnecessary since the potential interference such as tryptophan could be eliminated by the post-column derivatization protocol since the NBD-tryptophan derivative does not fluoresce. Aqueous methanolic mussel extracts as described above were injected directly into HPLC–PCD system, showed no interferences from tryptophan, and the chromatograms are similar with those from the SPE-treated samples.

3.2. Post-column derivatization optimization

The optimization of the derivatization reaction was a core study in our work. NBD-Cl (reagent 1) reacts with domoic acid similarly to secondary amines (Fig. 2). NBD-Cl itself is not fluorescent but when it reacts with amines (primary and secondary) a fluorescent product is produced. The reaction between the NBD-Cl and the DA yielding the fluorescent product is shown in Fig. 2. The product has a peak at 469 nm λ_{ex} and 529 nm λ_{em} based on a full fluorescence spectrum scan. The hydrolyzed product 4-hydroxy-7-nitro-benzo-2, 1,3-oxadiazole (NBD-OH) from the first reaction has weak fluorescence emission and loses its fluorescence intensity at pH about 1 [21]. In order to reduce the background fluorescence, hydrochloric acid was used as a second reagent (reagent 2) in our post-column system.

3.2.1. Reagent 1 – NBD-Cl concentration and pH

The first two variables studied in this work was the concentration of the reagent 1 and second the pH in which the post-column reaction takes place. The magnitude of fluorescence response of 0.2 ppm DA was examined as a function of both the NBD-Cl concentration and pH. As it is shown in Fig. 3, the optimum concentration for the first reagent (NBD-Cl) in methanol, buffered at pH 10.0 with boric acid was 9.0 mM. Increasing the concentration of the reagent further did not result in any further increase in the detector's response (see Fig. 3A). When the pH of the NBD-Cl reagent was

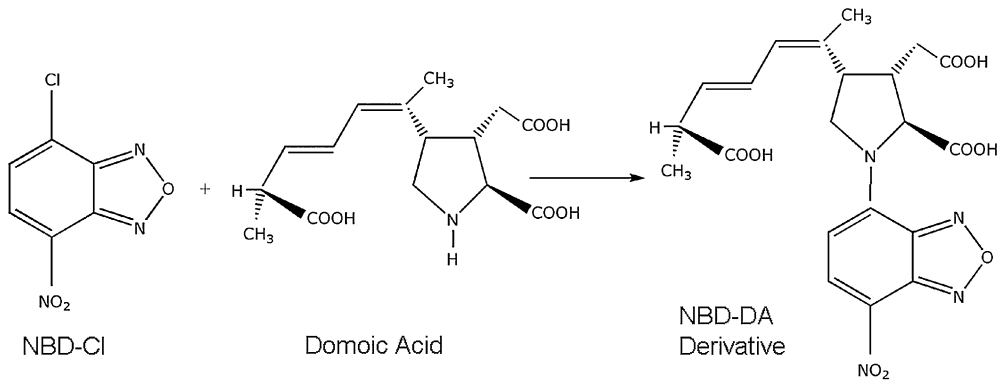

Fig. 2. Chemical reaction between NBD-Cl and DA.

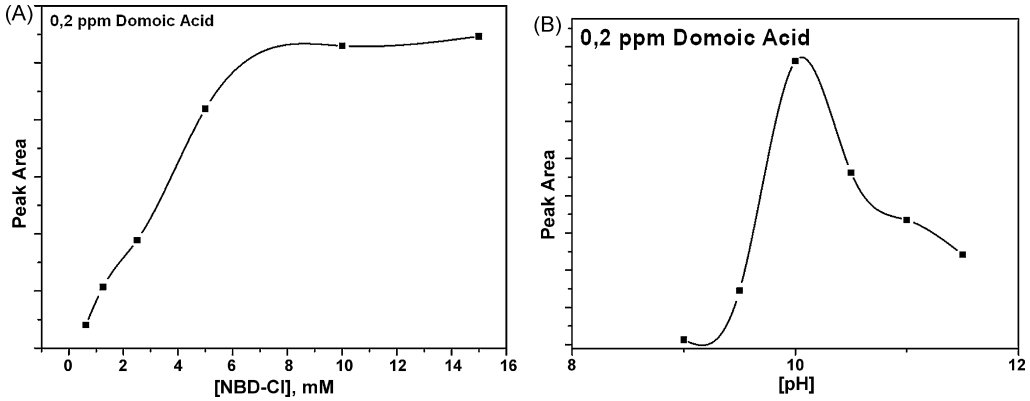

Fig. 3. (A) Effect of NBD-Cl concentration in reagent 1 to detector's response. (B) Effect of pH in reagent 1 to detector's response.

varied, a maximum peak area and height was reached at a value of pH 10.0 (see Fig. 3B). The pKa value of the amine in DA's pentameric ring is 9.8, and therefore, a borate buffer was used to study the pH effect on the reaction of DA and NBD-Cl. Boric acid was adjusted to different pH values, with the use of sodium hydroxide. A buffer concentration study from concentration 0.032 mM up to 0.2 mM showed no significant effect on the detector response. Therefore, 0.065 mM was used as the optimum buffer concentration, which can provide adequate buffer capacity to reagent 1, as well as to avoid at the same time precipitation problems of the borate buffer in the PCD.

3.2.2. Reagent 1 – type and organic solvent

NBD-Cl was dissolved in 100% methanol. Different types of organic solvents were studied and MeOH was proven to be the best solvent giving the best fluorescent signal. Because of the hydrolysis of NBD-Cl to NBD-OH water was not used as a solvent. Studies decreasing the percentage of organic solvent to 50% using 50% of water caused a decrease in detector's response.

3.2.3. Reagent 1 – coil volume

Subsequently the reaction coil volume of reagent 1 was studied. The volume of the first reagent coil played an important role in the detection of 2 ppm DA with NBD-Cl. Reagent coils from 500 μL to 1500 μL were tested and the best results were achieved with a 1500-μL coil as presented in Fig. 4A. Further increase of coil volume was not tested since this would increase the band broadening of the analytes. In the course of this study the effect of smaller internal diameter (i.d.) reagent coil tubing to the peak shape and detector's response of the analytes were also examined in order to determine whether the large coil volume of reagent 1 had an effect on the band broadening of the analytes. For this a smaller i.d. tubing was used while maintaining the same length to determine if this will improve the shape of the chromatographic peaks. A smaller i.d. tubing of 0.007 in. was used and compared with the 0.010 in. i.d. which was normally used for all the experiments. The total volume of the reagent coil using the same length of the smaller i.d. tubing (0.007 in.) resulted in less volume than the one with 0.010 in. It was finally determined that the smaller i.d. tubing did not improve the

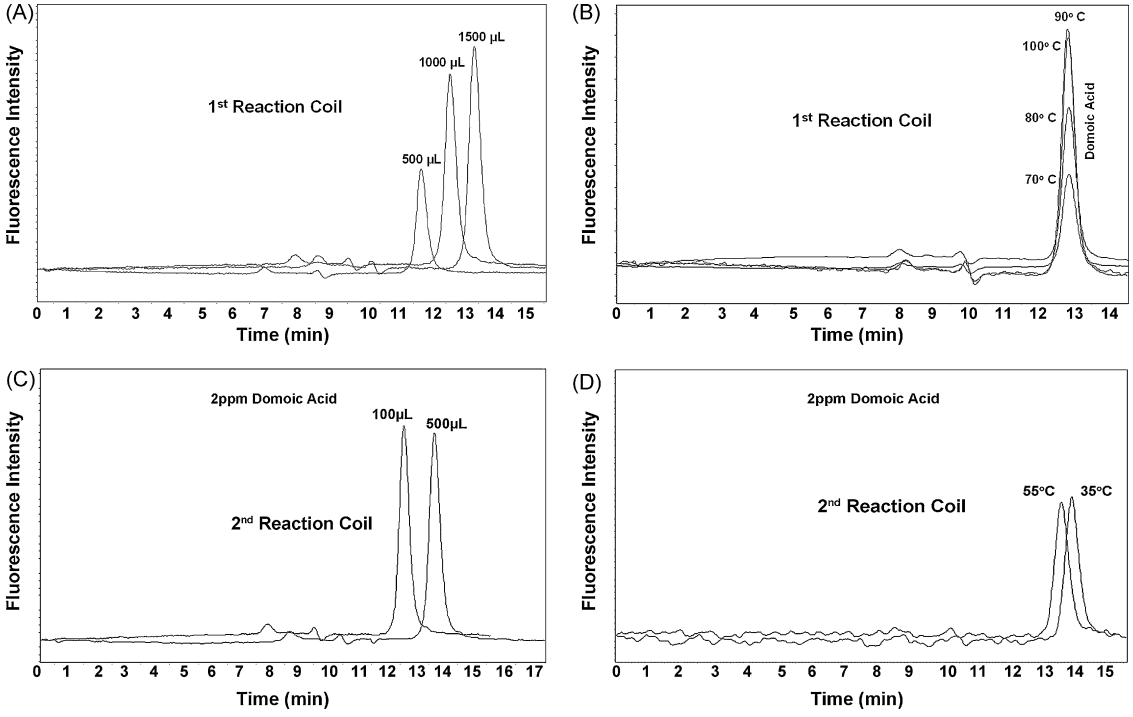

Fig. 4. (A) Effect of reagent coil 1 volume. (B) Effect of temperature in reagent coil 1. (C) Effect of reagent coil 2 volume. (D) Effect of temperature in reagent coil 2.

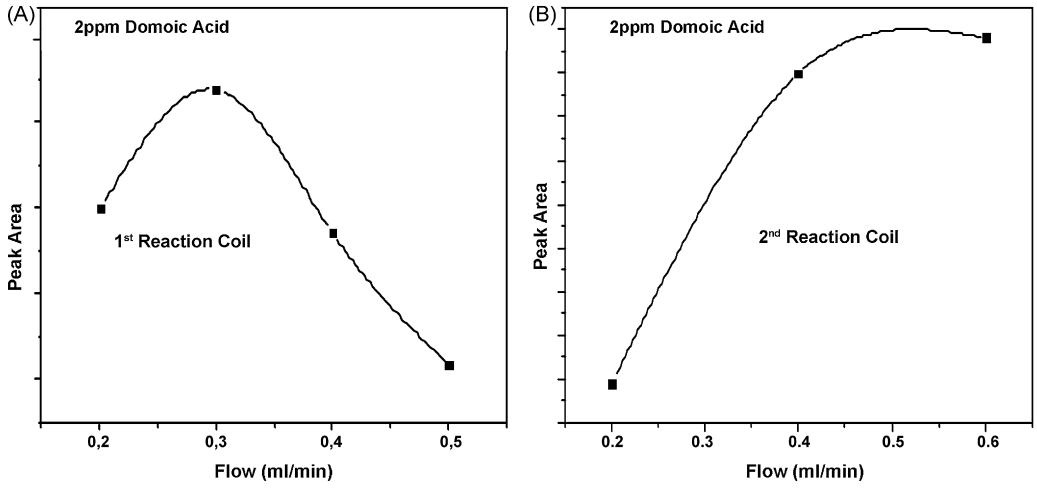

Fig. 5. (A) Effect of flow rate reagent 1. (B) Effect of flow rate reagent 2.

peak intensity and shape of the analytes leading to the conclusion that the 1500 μL volume with 0.010 in. i.d. is optimum for the completion of the reaction. Therefore, tubing with i.d. of 0.010 in. was used for all subsequent experiments.

3.2.4. Reagent 1 – temperature

The reaction of the NBD-Cl with DA is rather slow at room temperature. Heating of the first reaction coil was found essential in order to improve the kinetics of the reaction shown in Fig. 2. Different temperatures were studied and a summary of these data are shown in Fig. 4B). The temperature of the first reaction coil was found to be optimized at 90 °C. Increasing the temperature above 90 °C resulted in a decrease of the fluorescent signal.

3.2.5. Reagent 2 – type of acid type and organic solvent composition

Due to the high concentration of water in the mobile phase during the mixing of the NBD-Cl with the eluent there is subsequent NBD-OH formation which contributes to the background fluorescence. Therefore, an acidic reagent is essential for the reduction of the background fluorescence signal. Acetic, formic and hydrochloric acids were evaluated in concentrations range from 0.1 M to 1.5 M. The optimum acid for the reagent 2 was 1 M HCl, which was proven to be the most effective to destruct the fluorescent signal of the NBD-OH, the hydrolysis product of NBD-Cl. Subsequently the type of solvent was studied too. Several solvents were tested in the reagent 2 such as cellosolve, methanol, acetonitrile, acetone, ethanol, 2-propanol and ethylacetate. The highest fluorescence signal of the derivative between DA and NBD-Cl was obtained when ethyl acetate was used.

3.2.6. Reagent 2 – coil volume

The volume of the second reaction coil did not make any significant difference in the detector's response. Two coil volumes 100 and 500 μL were tested without any significant difference (see Fig. 4C). Again since the second reaction is very quick, and thus higher coil volumes are not needed. A smaller coil volume was chosen in order to minimize band broadening due to post-column system.

3.2.7. Reagent 2 – temperature

The temperature of the 2nd reaction coil was also examined at two temperatures. There was no significant effect on the peaks of the derivatives of DA and the IS (see Fig. 4D). Since the destruction of NBD-OH takes place at room temperature there was no need for heating in the second reaction coil. Therefore, the second reagent coil was kept in ambient temperature since detection did not benefit from raising the temperature.

3.2.8. Reagents 1 & 2 – flow rates

Studies of the flow rates for the first two reagents indicated that a flow rate of 0.3 mL/min for the NBD-Cl reagent and 0.6 mL/min for the HCl reagent were the best. Higher flow rates of reagent 1 decreased the fluorescence signal of the 2 ppm DA since the reaction time provided by this system in order to complete the reaction are not sufficient. A higher flow rate of 0.6 mL/min was used for the second reagent since the destruction of NBD-OH is fast (see Fig. 5A and B).

3.2.9. Reagents 1 & 2 – mixing volumes

The effect of mixing volume in the mixing tees was also studied too. In the PCD system used (model Prometheus 300 Plus) there is the possibility to change the mixing volume in the two static mixers (mixing tees). Mixing volumes from 50 μL to 250 μL were examined. The results showed that the optimum mixing volume was proven to be 50 μL for both mixing tees which is in agreement with the used flow rates (<1 mL/min for eluent and reagents).

The reproducibility of the PCD system was checked with 10 successive injections of the standard 250 ppb DA and IS. The results of the multiple chromatographs are summarized in Fig. 6. The reagent

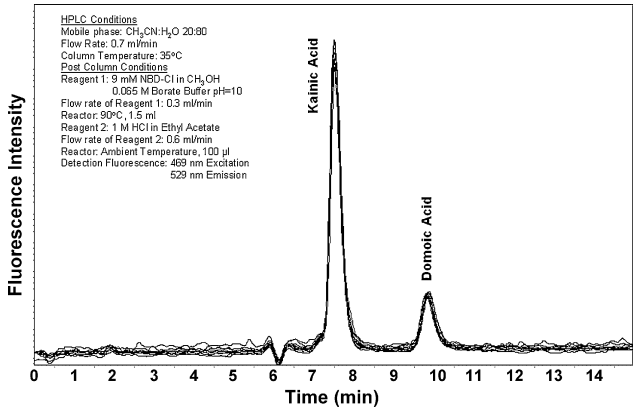

Fig. 6. Post-column reproducibility of the system with 250 ppb DA standard (multiple chromatograms) using 20:80 0.1% TFA in H_2O:Acetonitile as mobile phase.

Table 1
Standard addition recovery data on mussel extract.

μg domoic acid/g of mussel tissue added	Equivalent concentration of domoic acid added in mussel extract	μg domoic acid/g of mussel tissue found	Equivalent concentration of domoic acid found in mussel extract	Percent (%) recovery
0.375 μg/g	250 ppb	0.366 μg/g	244 ppb	97.6%
0.750 μg/g	500 ppb	0.735 μg/g	490 ppb	98.0%
1.50 μg/g	1000 ppb	1.49 μg/g	993 ppb	99.3%

was proven to be stable for at least 10 h based on the reproducibility of the analysis of the DA and IS.

3.3. Quantitative results

Using the above optimized chromatographic and optimum derivatization post-column conditions the method was tested for the analysis of DA in mussel samples. A non-contaminated mussel sample spiked with DA and IS was first used to test for any interferences. A typical chromatogram illustrating the resolution of the matrix, IS, and DA is shown in Fig. 7 where 17:83 acetonitrile:water was used to improve the resolution between the matrix and IS. As shown in this figure the matrix did not introduce any other interferences neither it affected the resolution of the IS and DA peaks, while the reproducibility was excellent.

A series of calibration standards from 50 ppb up to 1.5 ppm DA and 250 ppb IS in real sample matrix were then evaluated, using 100 μL injection volume. The calibration curve showed linearity from 50 ppb to 1500 ppb with correlation coefficient (R^2) of 0.9994. The least square analysis for the line gave the equation $y = 1.18 \times 10^{-3}x - 8.44 \times 10^{-4}$. The limit of quantitation (LOQ) of this method for DA was determined to be 50 ppb while its limits of detection (LOD) of S/N 3/1 was measured to be 25 ppb.

Standard addition recovery experiment was studied using DA and IS that were spiked into mussel tissue at 0.375 μg/g, 0.750 μg/g and 1.50 μg/g levels that corresponds to final concentration of domoic acid in the mussel extract before injection to 250 ppb, 500 ppb and 1000 ppb, respectively. Recoveries were calculated to be excellent, ranging from 97.6% to 99.3%. A summary of these results are listed in Table 1. The average recovery of DA from CRM-ASP-Mus-b standard material using the proposed SPE cleanup was 97.4% ($n = 5$). These data proves that indeed the proposed sample preparation in conjunction with the fluorescence detection following a post-column detection produce accurate and reproducible recoveries of DA in mussel samples. The precision of the method was checked for 10 consecutive injections at a level of 250 ppb DA and 250 ppb IS; the RSD was calculated to be at the very low level of 1.9%.

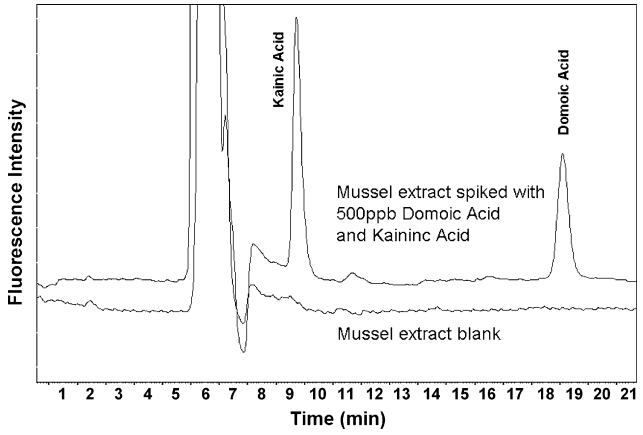

Fig. 7. Blank mussel extract without DA and IS-mussel extract with 500 ppb DA and IS using 17:83 0.1% TFA in H_2O:Acetonitile as mobile phase.

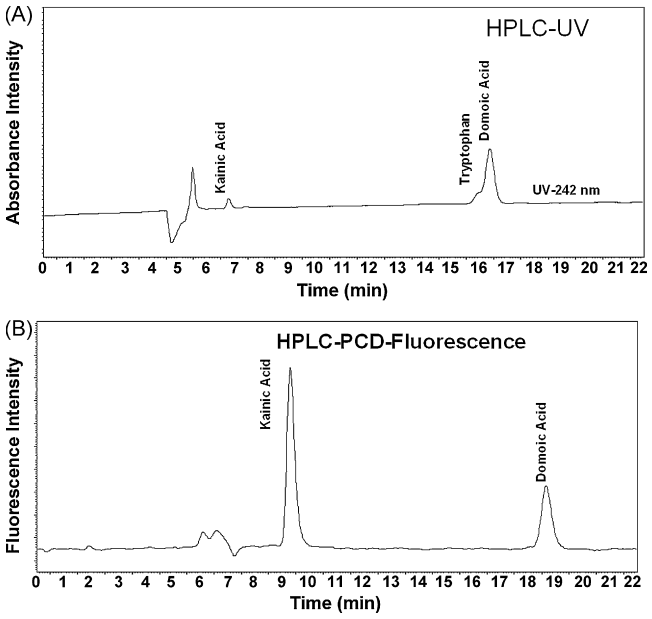

Fig. 8. Comparison of (A) HPLC-UV method and (B) HPLC-PCD-fluorescence method.

Although the LC-UV method offers adequate quantitation limits according to the current regulatory values more sensitive assays such as the proposed LC-post-column derivatization using fluorescence detection are always useful since the regulatory values could be reduced in the future based on new toxicity data.

It is thus concluded that the chromatographic analysis of DA on a reverse phase column requires acidic conditions can be achieved without any peak broadening and tailing. The problem reported in previous publication [5,22] in which tryptophan elutes near domoic acid in acidic pH and it thus usually interferes with the analysis of domoic acid is not a problem with the proposed method. Tryptophan which is present in shellfish matrix reacts with NBD-Cl yielding a non-fluorescent product overcoming this problem, thus its presence does not generate a problem here. As shown in Fig. 8A (without post-column derivatization) using a reverse phase column and monitoring with UV detection at 242 nm the tryptophan peak interferes with the DA peak. When PCD is used and detection is by fluorescence (see Fig. 8B) then no interference due to tryptophan is observed.

4. Conclusion

Our new method using post-column derivatization with fluorescence detection is the first successful implementation of post-column derivatization technique in DA determination using an IS. It offers a low cost alternative to selective MS techniques and a faster and automated replacement for pre-column derivatization techniques, since it overcomes the matrix co-eluted compounds that might interfere with DA with the fact that this low level analysis

can be performed on a relatively standard HPLC-PCD system. Simple extraction procedure using aqueous methanolic solutions in mussel tissues can be applied, without interferences from tryptophan, providing similar chromatograms with those from the SPE-treated samples. Higher sensitivity is obtained due to the use of the sensitive fluorescence detection, together with higher selectivity over that of the known HPLC-UV methods. The absence of the fluorescence of the product of the NBD-Cl with tryptophan is also one of the major advantages of the method. The post-column derivatization method using fluorescence detection with NBD-Cl provides cleaner chromatograms free of interferences than the up to now reported pre-column method separating domoic acid from internal standard using standard sample preparation protocols reported above. This method has detection limit (LOD) for DA in mussel of 37.5 μg/kg as the reported LOD of 500 μg/kg with the UV method [23] and the maximum amount of 20 mg/kg which is allowed by European Union (EU) Legislation [24]. It provides reproducible results with sufficient sensitivity to detect domoic acid in shellfish. This precise method is used for monitoring DA in mussels and it can be applied to seawater and phytoplankton samples too, where lower levels of DA are present.

Acknowledgement

This research project was co-funded by the European Union-European Social Fund and National Resources-EPEAK II.

References

[1] M.A. Quilliam, J.L.C. Wright, Anal. Chem. 61 (1989) 1058A.
[2] C.J. Bird, J.L.C. Wright, World Aquac. 20 (1989) 40.
[3] J.C. Wekell, E.J.J. Gauglitz, H.J. Barnett, C.L. Hartfield, D. Simons, D. Ayres, Nat. Toxins 2 (1994) 197.
[4] Z. Amzil, J. Fresnel, D. Le Gal, C. Billard, Toxicon 39 (2001) 1245.
[5] P. Vale, M.A.M. Sampayo, Toxicon 39 (2001) 893.
[6] P. Hess, S. Gallacher, L.A. Bates, N. Brown, M.A. Quilliam, J. AOAC Int. 84 (2001) 1657.
[7] S.S. Bates, C.J. Bird, A.S.W. de Freitas, R. Foxall, M. Gilgan, L.A. Hanic, G.R. Johnson, A.W. McGulloch, P. Odense, R. Pocklington, M.A. Quiliam, P.G. Sim, J.C. Smith, D.V. Subba Rao, E.C.D. Todd, J.A. Walter, J.L.C. Wright, Can. J. Fish Aquat. Sci 46 (1989) 1203.
[8] K.R. Buck, L. Uttal-Cooke, C.H. Pilskaln, D.L. Roelke, M.C. Villac, G.A. Fryxell, L. Ciflintes, F.P. Chavez, Mar. Ecol. Prog. Ser. 84 (1992) 293.
[9] T.M. Perl, L. Bedard, T. Kosatsky, J.C. Hockin, E.C.D. Todd, R.S. Remis, New Engl. J. Med. 322 (1990) 1775.
[10] D.F. Wozniak, G.R. Stewart, J.P. Miller, J.W. Olney, Exp. Neurol. 114 (1991) 250.
[11] M.A. Quilliam, P.G. Sim, A.W. McCulloch, A.G. McInnes, Int. J. Environ. Anal. Chem. 36 (1989) 139.
[12] R. Pocklington, J.E. Milley, S.S. Bates, C.J. Bird, A.S.W. de Freitas, M.A. Quilliam, Int. J. Environ. Anal. Chem. 38 (1990) 351.
[13] T. Sun, W.H. Wang, J. Agric. Food. Chem. 47 (1999) 4678.
[14] K.J. James, M. Gillman, M. Lehane, A. Gago-Martinez, J. Chromatogr. A 871 (2000) 1.
[15] A. Furey, M. Lehane, M. Gillman, P. Fernandez-Puente, K.J. James, J. Chromatogr. A 938 (2001) 167.
[16] Y. Watanabe, K. Imai, Anal. Chem. 55 (1983) 1786.
[17] H. Yoshida, T. Sumida, T. Masujima, H. Imai, J. High Resoult. Chromatogr. Chromatogr. Commun. 5 (1982) 509.
[18] M.R.L. Stratford, R.R. Watfa, J.C. Murray, S.G. Martin, J. Chromatogr. 526 (1990) 383.
[19] M.A. Quilliam, J. AOAC Int. 78 (1995) 555.
[20] M.A. Quilliam, M. Xie, W.R. Hardstaff, J. AOAC Int. 78 (1995) 543.
[21] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.
[22] A. Lopez-Rivera, B.A. Suarez-Isla, P.P. Eilers, C.G. Beaundry, S. Hall, M. Fernandez Amandi, A. Furey, K.J. James, Anal. Bioanal. Chem. 381 (2005) 1540.
[23] J.F. Lawrence, C.F. Charbonneau, C. Menard, J. Chromatogr. 462 (1989) 349.
[24] European Commission, Council Directive 91/492/EEC of 15 July, Off. J. L 268 (1991) 1.