ELSEVIER

Journal of Chromatography A, 764 (1997) 223–231

JOURNAL OF CHROMATOGRAPHY A

Optimization of sample cleanup procedure for determination of diarrhetic shellfish poisoning toxins by use of experimental design

Brit Aase*, Astri Rogstad[1]

Norwegian College of Veterinary Medicine, Department of Pharmacology, Microbiology and Food Hygiene, P.O. Box 8146 dep, N-0033 Oslo, Norway

Received 6 June 1996; revised 23 October 1996; accepted 23 October 1996

Abstract

In routine monitoring of diarrhoeic shellfish poison (dinophysistoxin-1 and okadaic acid) there appeared to be an inconsistency between the mouse bioassay and existing chemical analysis based on liquid chromatography. The sample cleanup procedure has been subject to minor modifications in an effort to overcome the problem. However, further studies have appeared necessary and in this study all experimental factors that can influence the sample cleanup using solid-phase extraction columns prior to the LC analysis have been evaluated by use of experimental statistical design in order to understand the effect of the various factors and to optimize the conditions for recovery of the toxins. Based on our experiments we suggest using a solid-phase extraction silica column of 100 mg in the sample cleanup procedure and washing solvents composed of dichloromethane instead of chloroform to minimize the effect of stabilizing alcohol. It is sufficient to apply 7.5 ml hexane–dichloromethane (1:1) to the column in the first washing step and 2.5 ml of dichloromethane in the final washing. Elution is complete by use of 2.5 ml chloroform with 3.5% methanol. Toxic shellfish tested by this procedure confirmed the mouse bioassay.

Keywords: Sample preparation; Shellfish; Experimental analysis; Toxins; Okadaic acid; Dinophysistoxin; Anthryldiazomethane

1. Introduction

Incidents of diarrhoea from eating mussels have occurred in Norway since the late 1960s. Since 1984 diarrhoeic shellfish poison (DSP) events were confirmed, and the toxin source was mainly species of dinoflagellates (*Dinophysis* spp., *Prorocentrum* ssp.) [1]. This has restrained development of commercial mussel production. The main toxins of the DSP group located along the Norwegian coast are okadaic acid (OA) and dinophysistoxin-1 (DTX1), but yessotoxin has also been detected [2]. The intoxication of humans after intake of the two former toxins is known, whereas the implications for human health after intake of yessotoxin are not clear. Thus, a surveillance program has been established to give warnings to people against picking wild mussels along the southern and western coast of Norway and to continuously alert the commercial mussel farms to have their harvest monitored for toxicity.

Presence of toxins has mainly been demonstrated by the mouse bioassay [3]. Continuous follow-up of

*Corresponding author.

[1] Permanent address: Nycomed Imaging AS, P.O. Box 4220 Torshov, N-0401 Oslo, Norway.

PII S0021-9673(96)00938-7

the results of the biological test has been performed by application of the cleanup and chromatographic method of Lee et al. [4] using 9-anthryldiazomethane (ADAM) as derivatizing reagent. The Lee method is being applied in most control laboratories world wide. However, minor modifications have been published to avoid loss and improve recovery of the toxins in the pretreatment method.

Ileby and Fiksdahl [5] reported reduced recovery of DTX1 when purifying the toxin derivative on the silica solid-phase extraction (SPE) column. Some of the washing fraction had to be collected to obtain optimal recovery of the toxin. The sample cleanup of the derivatized toxin on the SPE column was modified by Stabell et al. [6] by introducing dichloromethane, acetone and acetonitrile instead of chloroform and methanol. However, Stabell admitted that some loss of DTX1 occurred when washing the silica column with dichloromethane–acetone (9:1). Pereira et al. [7] investigated the influence of alternative solvents on the extraction procedure. Isopropanol was introduced for homogenization and combined ethyl acetate and hexane in the aqueous sodium sulphate solution for further extraction of the homogenate. Pereira et al. [7] have also applied Stabell's method for pretreatment of the ADAM derivative and studied the influence of solvents and the concentration of the ADAM reagent on the reliability of the method. Automated column switching in combination with HPLC has also been used to reduce loss of toxin in the sample pretreatment [8]. The most recent and complete study for evaluation of the liquid chromatography method combined with mass spectrometry or fluorimetric detection has been performed by Quilliam [9].

Several derivatization reagents have also been tested to get a cleaner and more stable derivatized product and to improve the sensitivity of the Lee method. Dickey et al. [10] introduced 1-bromoacetylpyren, whereas Zonta et al. [11] applied 9-chloromethylanthracene. Shen et al. [12] have used 4-bromomethyl-7-methoxycoumarin. Akasaka et al. [13] used Pereira's method for sample homogenization and prepared the toxin derivative with 2,3-(anthracenedicarboximido)ethyl trifluoromethansulfonate. This new fluorescing derivative improved the sensitivity of the toxins considerably. Many of these reagents are, however, of limited interest to control laboratories as they are not commercially available or the detection sensitivity of the toxin derivatives is not improved compared to the ADAM derivative in the Lee method.

However, discrepancies between the results obtained by mouse bioassay and the HPLC method happened to occur in our laboratory when the pretreatment procedures of Lee or Stabell were applied and a search for losses of toxicity in the samples was therefore initiated. It appeared that the reproducibility of the analytical results was not acceptable when applying solvents from various suppliers. When using Stabell's method the recovery appeared to be dependent on the quality of the silica in the SPE column.

2. Experimental

2.1. Sampling of shell fish, treatment, extraction and stability

Mussel samples were collected from two locations along the Norwegian coast. Samples containing mainly DTX1 were collected in Sogndal at the south-west coast, whereas samples with OA were from Fløde vigen at the south coast. The samples were used for preparation of test mixtures of OA and DTX1 for the analytical optimization work. Mussels were also harvested over a period of 9 months from a location in the Sognefjord at the south-west coast to study the seasonal variations of OA and DTX1. The shells were collected at a depth of 4 m.

The mussels were boiled, peeled and frozen at the harvest place in some cases and in our laboratory in other cases. The samples were either forwarded frozen or refrigerated when fresh. Before extraction the hepatopancreas (HP) was separated from the mussel meat, homogenized in an Ultra-Turrax (IKA, Staufen, Germany) and frozen. The sample size was normally about 50 g HP. In the case of large quantities of toxins large samples of mussels (100 kg) were collected.

Portions of 1 g HP were extracted using the extraction procedure published by Lee et al. [4]. According to the MUS-2 (mussel tissue reference material for DSP toxins) documentation [14] this is a dispersive extraction which ends with approximately

the correct result. The HP sample was blended with 4 ml 80% methanol for half a min, centrifuged and 2.5 ml supernatant was transferred to another glass tube for further extraction. The extract was washed twice with 2.5 ml hexane which was discarded. Following addition of 1 ml water the sample solution was extracted twice with 4 ml chloroform. The combined chloroform extracts were evaporated just to dryness under nitrogen and quickly redissolved in 1 ml methanol.

The shelf-life stability of this methanol solution of OA and DTX1 has been tested over a period of 1 month. Storage temperatures were 4°C (fridge) and −20°C (freezer). The storage time for the methanol extract in freezer was extended to 8 months. Sampling was performed after 1 day, 1 week, 1 month and 8 months. The samples were derivatized and pretreated on SPE columns as described below.

2.2. Reagents and glassware

The solvents used for the clean-up procedure were chloroform, methanol and dichloromethane, all of analytical grade from Merck (Darmstadt, Germany). Dichloromethane from Rathburn (Walkerburn, UK) and chloroform from Lab-Scan (Dublin, Ireland) were also included in the studies. Solvents used for the HPLC analyses were acetonitrile and methanol of LC quality and supplied by Romil (Cambridge, UK). The water was Milli-Q cleaned. The derivatizing reagent ADAM was purchased from both H.L. Biotech (Gothenburg, Sweden) and Serva (Heidelberg, Germany); these were of almost the same quality. The reagent was stored at −20°C as dry substance in small portions of 10 mg and was handled with care to avoid exposure to humidity. The tube of reagent was always acclimatised in a desiccator for 1 h before opening. The methanol solution of ADAM (0.2%, w/v) was prepared immediately prior to use. To overcome the problem of poor solubility in methanol ADAM was initially dissolved in one drop of acetone (20–40 μl).

Following washing the glassware was heated to 300°C over night to burn off impurities.

The analyses for the experimental designs were mainly based on the external standard method, whereas deoxycholic acid (DOCA) from Fluka (Buchs, Switzerland) was used as internal standard when monitoring the toxin levels. DOCA was dissolved in methanol to a concentration of 1.0 μg/ml and this solution was used as solvent for ADAM (0.2%, w/v). OA supplied by Marine Analytical Chemistry Standards Program (Halifax, Canada) was used as calibration standard and was prepared in blank mussel extracts in five concentrations from 5 to 100 ng/μl. Each standard was prepared in triplicate. A small quantity of purified DTX1 was a gift from T. Yasumoto and was used for qualitative testing. A MUS-2 sample containing both OA and DTX1 was used for quantitative control of our method.

2.3. Procedure for derivatization with ADAM and solid-phase cleanup (modified method)

Mussel extract (50 μl) and ADAM solution (50 μl) containing DOCA were transferred to amber PTFE-lined screw-cap vials. Following vortex mixing and ultra sound sonication (5 min) the samples were placed in darkness for 1 h at 30°C. The reaction mixture was carefully taken to dryness using nitrogen and immediately redissolved in 0.5 ml dichloromethane–hexane (1:1).

Silica SPE columns from Varian (Harbor City, CA, USA) were used for the cleanup procedure in combination with a SPE manifold from Supelco (Bellefonte, PA, USA). Following conditioning of the column the sample was applied. The column was washed in two steps, first with dichloromethane–hexane (1:1, washing solvent 1) and then with dichloromethane (washing solvent 2) and eluted with chloroform–methanol (96.5:3.5). Initially, chloroform (Lee method) was used in all steps of the SPE procedure instead of dichloromethane. In the final version of the method dichloromethane with 0.1% alcohol from Rathburn and chloroform from Merck were applied.

The eluates were taken carefully to dryness using nitrogen (g) and immediately redissolved in 200 μl methanol using sonication. The methanol fraction was filtered through a Costar spin-X filter of 0.2 μm (Cambridge, MA, USA). The samples were analyzed by HPLC within 24 h.

SPE columns from Millipore (Bedford, MA, USA) and Mallinckrodt (Hennef, Germany) were tested.

2.4. HPLC-analysis

The LC system consisted of an LKB 2249 gradient pump (Pharmacia, Bromma, Sweden), an LKB 2157 autosampler and a Perkin Elmer LS-1 fluorescence detector (Norwalk, CT, USA) with a 365 nm excitation filter and emission wave length of 412 nm. The separation was carried out using 5 μm Merck LiChrospher 100 RP18 column (250×4 mm), precolumn (4×4 mm) and isocratic elution with acetonitrile–water (82:18) at a flow-rate 1.1 ml/min. The system was equipped with an in-line filter of 0.5 μm from Upchurch Scientific (Oak Harbor, WA, USA).

The injection volume was 20 μl. As a rule the derivatized samples were injected within 24 h when stored at room temperature or within 48 h when stored at −20°C.

3. Experimental design and optimization

The development and optimization of the SPE pretreatment of the HP extracts were carried out in three steps using statistical experimental design. In a final step optimization of the derivatization reaction was performed. For the statistical calculations the software STATGRAPHICS Plus for Windows, Version 1 (Manugistics, Rockville, MD, USA) was used.

In the initial step it was searched for the most important variables to optimize the recovery of OA and DTX1 in the SPE cleanup. Six variables were included in the screening design, being the amount of silica in the extraction column, the amount of the washing solvent 2 (chloroform–methanol) and the concentration of methanol in the washing solvent (including alcohol in the chloroform). It was assumed that washing solvent 1 (hexane–chloroform) was not going to have any effect on the toxin recovery. Vacuum was applied to the SPE-manifold and the speeds of both the washing procedure and elution procedure were controlled and included as variables in the design. Finally, the methanol concentration in the eluent was included, whereas the amount of eluting agent was kept constant at 5 ml in these experiments. The factors and their experimental domain are listed in Table 1. Responses were

Table 1
Variables and their domain used in the various steps of the optimization procedure

Design/Domain	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
Screening design	*Amount of silica in column (mg)*	*Amount of wash solvent 2 (ml)*	*Conc. of alcohol in wash solvent 2 (%)*	*Speed of washing (ml/min)*	*Eluting solvent; methanol added to chloroform (%)*	*Speed of elution (ml/min)*
High value	1000	5	1.5	2.5	5	2.5
Low value	100	1	1	0.5	2	0.5
Design I	*Amount of wash solvent 2 (ml)*	*Eluting solvent; methanol added to chloroform (%)*	*Speed of elution (ml/min)*			
High value	2	5	2.5			
Low value	0.5	1	0.5			
Design II	*Amount of washing solvent 1 (ml)*	*Amount of washing solvent 2 (ml)*	*Amount of eluting solvent (ml)*			
High value	10	5	5			
Low value	5	1	1			
Design III	*Conc. of ADAM (%)*	*Derivatization temperature (°C)*	*Derivatization time (min)*			
High value	0.3	50	120			
Low value	0.05	20	15			

peak height of OA and DTX1. The screening design was reduced to 19 experiments (quarter fraction) including three center points.

The most significant variable from the screening design was combined with the factors of the elution process, composition of eluting solvent and speed of the elution process. Three factors were combined in a full factorial design of 15 experiments including three center points and second order interactions to obtain the optimal values of the factors in combination (Design I in Table 1).

In the third step the chloroform in the washing solvents was replaced by dichloromethane. The co-influence of the washing and eluting solvents was studied in a full factorial design of 15 experiments including second order interactions (Design II in Table 1).

The optimization of the conditions for the derivatization reaction with ADAM was done by varying the amount of reagent and the reaction conditions (Design III in Table 1).

4. Results and discussion

4.1. Modification of Lee's method

The mouse bioassay is mainly being used for quality control of DSP-content in shellfish harvested along the Norwegian coast. However, the results have been tested regularly by use of Lee's or Stabell's HPLC-methods. Discrepancies between the two testing methods have occurred. Some initial testing of Lee's method using chloroform from two different suppliers gave inconsistent results of the toxin level in the mussel samples, see Table 2. It was therefore decided to perform a systematic study of all possible factors having influence on the method using experimental design.

The initial screening design (Table 1) showed that 100 mg Si (Varian) in the SPE-column was sufficient to retain the toxins on the column. Only a small quantity of pure chloroform, declared to contain 0.6–1% alcohol was required for column washing to separate the impurities from the analytes. However, a column with large amount of Si required more chloroform than applied in the design. In this case baseline separation was not obtained, see Fig. 1. The combined effect of the amount of Si and the washing solvent 2 is demonstrated in the contour plot in Fig. 2. When using vacuum on the SPE manifold the rate of washing solvent through the column hardly effected the level of contaminants, whereas increased

Table 2
Analytical results; a mixture of positive samples containing OA and DTX1

Method	OA (μg/g HP)	DTX1 (μg/g HP)
Our modified method	4.5	4.4
Lee's method		
Merck, K20286045	4.3	4.0
Merck, K21620645	4.8	4.1
Lab-Scan, 0735/5	3.9	1.1
Stabell's method	4.3	3.4

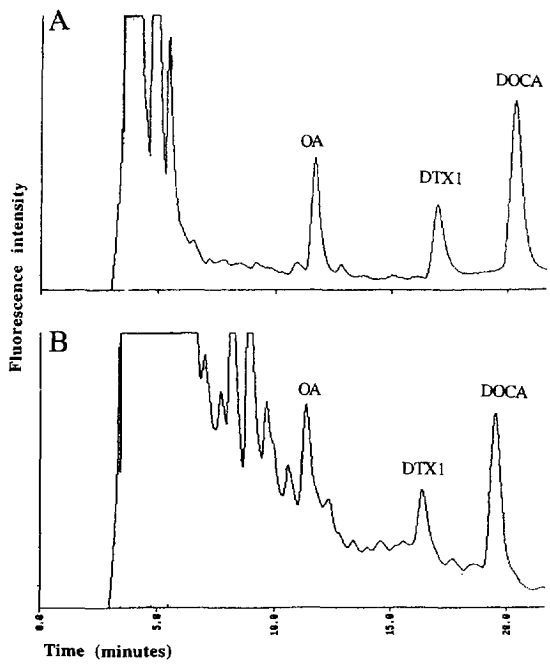

Fig. 1. Chromatograms obtained in the initial screening design using different amounts of Si in the SPE-column: A= 100 mg Si, 1 ml chloroform for washing, 2 ml chloroform–methanol (95:5) for elution; B= 1000 mg Si, 5 ml chloroform for washing, 5 ml chloroform–methanol (95:5) for elution.

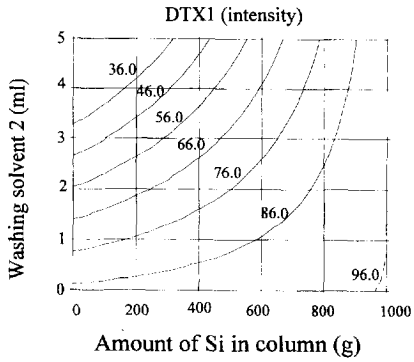

Fig. 2. Contours of estimated response surface for DTX1 showing the combined effect of Si amount in the SPE column and washing solvent 2 on the recovery; initial screening design.

elution speed gave a slightly reduced recovery of OA.

It is known that chloroform may contain varying amounts of alcohol. It was therefore of importance to study whether small variations had any influence on the retention of the toxins on the SPE column. Columns with 100 mg Si were chosen for the further studies of the sample pretreatment, as this will significantly reduce the cost of the sample cleanup.

The next step in the optimization work focused on the co-effect of alcohol in both washing solvent 2 (chloroform) and eluent in a full factorial design. It was assumed that optimal recovery of both DTX1 and OA in the cleanup procedure was possible by compromising the amount of solvent and the concentration of alcohol therein. The speed of the eluent through the column was also included as factor, see Design I (Table 1). The results show that only 0.5–0.8 ml of chloroform had to be applied to avoid loss of DTX1, indicating that small variations of alcohol concentration in the chloroform had a significant effect on the recovery. Consequently, the alcohol content in the supplied chloroform will have to be analyzed and kept constant when this solvent is being used in the sample cleanup. In some laboratories the alcohol content in chloroform has been routinely controlled [9,14]. This procedure is, however, considered too costly for a control laboratory monitoring large number of samples. Washing with a small volume of chloroform also had a positive effect on the recovery of OA. Eluting agent consisting of 3.5% methanol added to chloroform appeared to give optimal recovery of both analytes, which was little affected by the speed of eluent through the column.

Based on the above results it was therefore decided to change from chloroform to dichloromethane in the washing solvents to increase the robustness of the method. Dichloromethane from Rathburn contained 0.1% ethanol as stabilizer, whereas Merck supplied dichloromethane with 2-methylbutene. Both types were tested. The dichloromethane with alcohol washed out all the impurities from the column, whereas some of the impurities were retained on the column when using dichloromethane without alcohol. In the latter case the impurities were coeluted with the toxins.

In the final step of the optimization of the SPE cleanup for determination of DSP toxins we studied the co-influence of both washing solvents with the eluting solvent. Dichloromethane was applied in the washing solvents and chloroform in the eluting solvent. The optimum of washing solvent 1 was calculated to be 6–7 ml for OA and 9 ml for DTX1. Washing solvent 2 appeared to be very critical for optimal recovery of DTX1 and only 2–3 ml dichloromethane with 0.1% ethanol in combination with 2–3 ml eluent should be used, see Fig. 3. The optimal amount of washing solvent 2 for OA was not determined due to overlap of impurities in some of the chromatograms, but again 2–3 ml eluent was fully sufficient to obtain complete recovery. After repeated analyses it was concluded to use 7.5 ml washing solvent 1, 2.5 ml dichloromethane for washing solvent 2, and elute with 2.5 ml chloro-

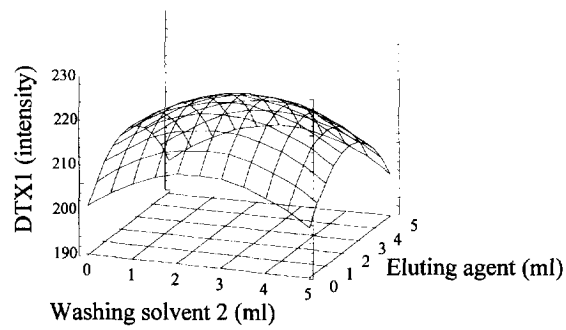

Fig. 3. Estimated response surface for DTX1 showing the co-effect of washing solvent 2 and eluting agent on the recovery; design II.

form–methanol (96.5:3.5) for routine analysis in our laboratory.

4.2. Preparation of ADAM derivative

Considering that the ADAM reagent is very expensive it was questioned whether it will be possible to optimize the preparation of fluorescing derivatives of OA and DTX1 by change of reaction conditions. The limit values in the design are listed in Table 1 (Design III). Initially, it was assumed possible to reduce the concentration of ADAM and the high value was set to 0.1%. The experimental results and calculations indicated that the optimum of the derivatizing reaction was not reached with 50 μl 0.1% ADAM for 1 h at 50°C. In a repeated investigation the high value of ADAM was increased to 0.3%. Fifteen experiments were performed and the results demonstrated that the concentration of ADAM is the most important of the three factors. The formation of both toxin derivatives was optimal with 0.2% ADAM. Increase of reaction time and temperature appeared to have a minor influence. Probably there was a slight degradation either of the toxin derivative or the ADAM reagent at 50°C and 120 min. However, it was observed that at a high concentration of ADAM (0.2% and more) accelerated degradation was obtained even at room temperature and impurity peaks appeared in number and intensity which slightly overlapped the OA and DTX1 peaks. The results of the statistical analysis were thus difficult to interpret. Very little was gained by increasing the ADAM concentration beyond 0.1%, which mainly caused enhanced expenses.

4.3. Application of modified method

The modified SPE procedure was tested using several batches of columns from the three suppliers Varian, Millipore and Mallinckrodt. The reproducibility among lots was acceptable. No significant differences were observed between columns from the three manufacturers, indicating the robustness of the method. Lot variations of dichloromethane had no influence and there was no need to either analyze the content of ethanol in the dichloromethane or distil off the ethanol to control the alcohol content in the washing solvents [9,14]. The repeatability of the method was about 1.5–3.5% at a level of 1 μg OA/g HP and about 0.5–3.0% for DTX1 at a content of 0.5 μg/g HP. The modified procedure was also compared with both Lee's and Stabell's methods, see Table 2.

The modified cleanup procedure was applied for determination of the toxin level in mussels harvested at the Norwegian south-west coast. The results are listed in Table 3 and a chromatogram of one of the samples is shown in Fig. 4. The results show that this location had a dominance of DTX1 and that the content of OA in the blue mussels was very limited. During spring and summer the toxin level was low and constant. However, in August the content of both toxins increased significantly and the toxin level stayed high until the end of the year. By the application of the modified cleanup procedure the toxin levels determined by HPLC were consistent with the levels determined by the mouse bioassay [15].

4.4. Shelf life stability of the toxins

Shelf life stability testing of the ADAM derivative of the toxins after cleanup on an SPE column with silica by Lukas et al. [16] showed it to be stable for 5 days at −20°C. Stabell et al. [6] performed stability testing of the derivatives for 7 days at 4°C and

Table 3
DSP-level in blue mussels harvested at the south-west coast of Norway in 1994

Harvesting time (month/week number)	DTX1 (μg/g HP)	OA (μg/g HP)
March*	3.0	0.2
April*	1.1	<0.1
May*	1.0	0.1
June*	1.1	0.1
July*	1.0	0.1
August*	1.5	<0.1
Week 35 (Aug. 28)	7.0	0.8
Week 37	9.9	0.6
Week 39	16.9	2.1
Week 41	19.0	1.9
Week 43	19.3	2.2
Week 45	18.0	2.5
Week 47	15.2	1.8
Week 49 (December 8)	9.8	0.8

* Average of two samplings at a time lag of 2 weeks.

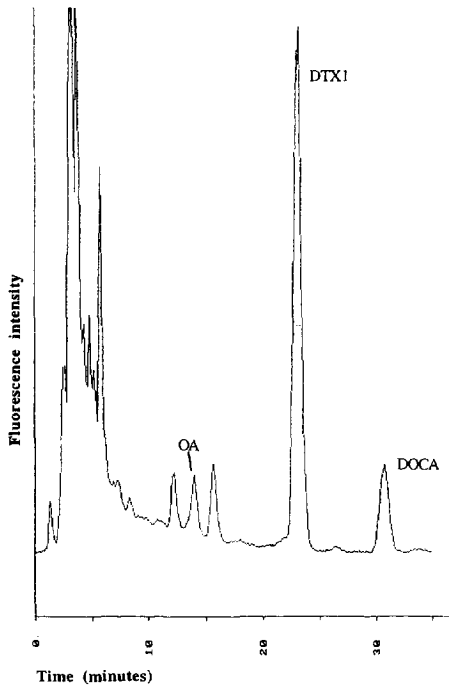

Fig. 4. Chromatogram of a sample of blue mussels harvested in week 45, 1994 on the south-west coast of Norway.

concluded that no breakdown of the ADAM derivatives was observed. In the documentation from Ileby and Fiksdahl [5] it has been mentioned that both toxins and their derivatives are prone to degradation even when stored under cold conditions. Their investigation included, however, only storage of the toxin derivatives. They found that the ADAM derivative of DTX1 is less stable than that of OA. After storage for 4 days at −30°C an additional chromatographic peak occurred and the DTX1 peak decreased in intensity. These inconsistencies prompted us to perform stability testing of the toxin extract prior to derivatization and column pretreatment. Storage of the tissue extract in methanol for 8 months at −20°C had no effect on the toxin level in the samples. However, storage for 1 month at 4°C caused degradation of the dry product. The content of OA was reduced by 25% and that of DTX1 by 18%.

5. Conclusions

By use of experimental design studies the effect of solvents on the sample cleanup of derivatized OA and DTX1 has been investigated. The results showed that small quantities of alcohol in the solvents applied to the SPE columns have a significant effect on the recovery of toxins. Lee's method has been modified by introducing 100 mg silica column for the sample pretreatment and applying dichloromethane instead of chloroform to the washing steps of the solid-phase extraction procedure. The amount of washing solvents and eluting solvent have been reduced compared to Lee's method. The robustness of the modified method is good. Knowledge of the quality and specifications of the solvents is essential, as ethanol used for stabilization of chloroform and dichloromethane appeared to vary considerably in content between different manufacturers and among production lots. Lot variations of the small amount of alcohol (0.1%) present in dichloromethane appeared less critical than the lot variations of chloroform. However, low content of ethanol was essential for optimal purification of the sample without loss of toxins, whereas slightly higher content of ethanol in the chloroform appeared to wash out the toxins of the column and reduce the recovery.

Lee et al. [2] suggested the use of ADAM at a concentration of 0.1% in the derivatizing reaction and our investigation confirmed the conditions for this reaction. The suggested modifications of the Lee method will reduce the cost of the DSP analysis as being performed routinely in many control laboratories.

References

[1] H.P. van Egmond, T. Aune, P. Lassus, G.J.A. Speijers and M. Waldock, J. Natural Toxins, 2 (1993) 41–83.
[2] J.S. Lee, K. Tangen, E. Dahl, P. Hovgaard and T. Yasumoto, Nippon Suisan Gakkaishi, 54 (1988) 1953–1957.
[3] T. Yasumoto, Shokuhin Eiseigaku Zasshi, 31 (1981) 515–522.
[4] J.S. Lee, T. Yanagi, R. Kenma and T. Yasumoto, Agric. Biol. Chem., 51 (1987) 877–881.
[5] N. Ileby and A. Fiksdahl, in S.S. Bates and J. Worms (Editors), Proceedings of the First Canadian Workshop on Harmful Marine Algae, Canadian Technical Report of Fisheries and Aquatic Sciences, Moncton, 1712 (1989) 19–20.
[6] O.B. Stabell, V. Hormazabal, I. Steffenak and K. Pedersen, Toxicon, 29 (1991) 21–29.

[7] A. Pereira, D. Klein, K. Sohet, G. Houvenaghel and J.C. Braekman, in P. Lassus, G. Arzul, E. Erard, P. Gentien and C. Marcaillou (Editors), Harmful Marine Algal Blooms, Lavoisier, Intercept, 1995, pp. 333–338 and references cited therein.
[8] C. Hummert, J.L. Shen and B. Luckas, J. Chromatogr. A, 729 (1996) 387–392.
[9] M.A. Quilliam, J. AOAC Int., 78 (1995) 555–570.
[10] R.W. Dickey, H.R. Granade and F.A. Bencsath, in T.J. Smayda and Y. Shimizu (Editors), Toxic Phytoplankton Blooms in the Sea, Elsevier, Amsterdam, 1993, pp. 495–499.
[11] F. Zonta, B. Stancher, P. Bogoni and P. Masotti, J. Chromatogr., 594 (1992) 137–144.
[12] J.L. Shen, C. Hummert, G. Ganzlin and B. Lukas, in W. Baltes, T. Eklund, R. Fenswick, W. Pfannhauser, A. Ruiter and H.P. Thier (Editors), Proceedings of European Conference on Food Chemistry, B. Behr, Hamburg, 1991, pp. 821–827.
[13] K. Akasaka, H. Ohrui, H. Meguro and T. Yasumoto, J. Chromatogr. A, 729 (1996) 381–386.
[14] Marine Analytical Chemistry Standards Program, Reference Material Documentation: MUS-2, Halifax, Canada, version 1993, pp. 1–19.
[15] T. Aune, Ø. Strand, B. Aase, J. Weidemann, E. Dahl and P. Hovgaard, Abstracts of 7th International Conference on Toxic Phytoplankton, Sendai, Japan, 1995, p. 4.
[16] B. Luckas, C. Hummert, G. Thielert, J. Kirschbaum and A. Boenke, BCR information, EUR15339, European Commission, Brussels, 1994, pp. 1–85.