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Journal of Chromatography A, 943 (2001) 39–46

JOURNAL OF CHROMATOGRAPHY A

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# Matrix effect and correction by standard addition in quantitative liquid chromatographic–mass spectrometric analysis of diarrhetic shellfish poisoning toxins

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Received 31 October 2000; received in revised form 18 October 2001; accepted 22 October 2001

**Abstract**

An evaluation of the feasibility of liquid chromatography–mass spectrometry (LC–MS) with atmospheric pressure ionization was made for quantitation of four diarrhetic shellfish poisoning toxins, okadaic acid, dinophysistoxin-1, pectenotoxin-6 and yessotoxin in scallops. When LC–MS was applied to the analysis of scallop extracts, large signal suppressions were observed due to coeluting substances from the column. To compensate for these matrix signal suppressions, the standard addition method was applied. First, the sample was analyzed and then the sample involving the addition of calibration standards is analyzed. Although this method requires two LC–MS runs per analysis, effective correction of quantitative errors was found. 

*Keywords:* Standard addition; Shellfish poisoning toxins; Toxins

## 1. Introduction

To analyze diarrhetic shellfish poisons (DSPs), high-performance liquid chromatography (HPLC) with a fluorescence (FL) detection system is most often used [1–11]. In the HPLC–FL method, DSPs must be derivatized with fluorescence reagents. It is difficult to analyze various DSPs simultaneously by HPLC–FL, since different fluorescence reagents are necessary for derivatization. Derivatization of the DSPs with 9-anthryldiazomethane (ADAM) is commonly used for FL detection of some acidic toxins, for example, okadaic acid (OA), dinophysistoxins (DTXs) and pectenotoxin (PTXs) [2,3,6,9]. For yessotoxin (YTX), which is one of DSPs that occurs with OA and DTX1, a different fluorescent reagent must be used because of the lack of a carbonyl group [8]. Besides, the impurities involved in the fluorescence reagents must be separated before injection into the HPLC system.

On the other hand, liquid chromatography–mass spectrometry (LC–MS) using atmospheric pressure ionization (API) is a powerful tool for identification and quantitation in the pharmaceutical or bioanalytical fields. However, the ionization efficiency of API is greatly affected by coeluting matrix compounds [12–16]. The ion suppression was described by Kebarle and Tang [12], who showed that electrospray ionization responses of organic bases decreased with an increase in the concentrations of

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0021-9673/02/$ – see front matter 
PII: S0021-9673(01)01429-7

other organic bases. Matuszewski et al. stated that undetected coeluting matrix components may reduce the ionization efficiency of the analytes and cause poor reproducibility and accuracy [14]. The matrix effect is especially dependent on the degree of sample purification and the degree of chromatographic separation of the analytes from the sample matrix components on the analytical column.

LC–MS has both high sensitivity and selectivity, which makes it possible to determine DSPs by direct injection without derivatization. The identification and quantitation of shellfish poisons and their metabolites by LC–MS have been reported [17–25]. For example, Draisci et al. reported that LC–MS is particularly useful to give an indication of the distribution of mussel contamination by DSP toxins [20]. Quilliam showed that LC–MS is suitable for the quantitative analysis of DSP toxins without chemical manipulation [21]. However, little is known about the matrix effect by coeluting substances in shellfish extracts.

The purpose of the present work was to evaluate the degree of the signal suppression for DSPs by coeluting substances in the scallop extract and to correct the matrix effect. In this paper, the evaluation of four DSPs, i.e. OA, DTX1, YTX and PTX6 by LC–MS is described. These DSPs are sometimes detected in scallops in Japan.

## 2. Experimental

### 2.1. Reagents

Methanol of HPLC grade was purchased from Kanto (Tokyo, Japan). Ammonium acetate was purchased from Fluka (Buchs, Switzerland). OA, DTX1 and PTX6 were purchased from Wako (Osaka, Japan). YTX was from the Institute of Environmental Science and Research (Porirua, New Zealand). The molecular structures of the four diarrhetic shellfish poisons are shown in Fig. 1.

Stock solutions (100 mg/l) of individual shellfish toxin standards were prepared by dissolving in methanol. A mixed stock solution (10 mg each toxin/l) containing four standards was prepared from stock solutions of individual standards by mixing and diluting with methanol. Stock solutions were stored at under −30°C. Calibration standards were prepared by appropriate dilution of the mixed stock solution with methanol.

### 2.2. Apparatus

The LC–MS system consisted of a Hitachi (Tokyo, Japan) L-7100 pump with a low-pressure gradient unit, a Hitachi L-7200 autosampler and a Hitachi M-8000 mass spectrometer with a sonic

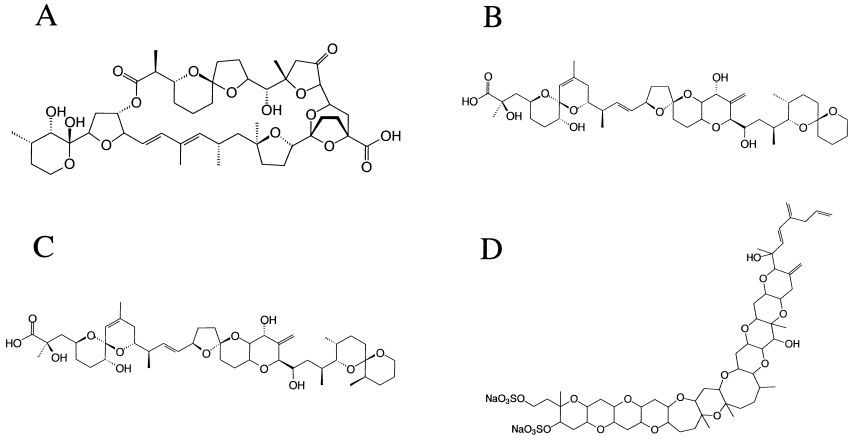


Fig. 1. Molecular structures of diarrhetic shellfish poisons. (A) Pectenotoxin-6 (PTX6); (B) okadaic acid (OA); (C) dinophysistoxin-1 (DTX1) and (D) yessotoxin (YTX).

spray interface (SSI) [26,27]. The HPLC column was an Inertsil ODS-2 (150×2.1 mm I.D., 5 μm) from GL Science (Tokyo, Japan).

### 2.3. Chromatographic conditions

Gradient elution was carried out using a binary gradient composed of solvent A (1 m*M* ammonium acetate solution in water) and solvent B (methanol) according to the following program: linear gradient from 40 to 100% B from 0 to 20 min, maintaining 100% B from 20 to 30 min, returning to the initial condition (40% B) at 30 min and maintaining this condition to 40 min. The flow-rate was set to 0.2 ml/min and 10 μl of sample were injected onto the column at the room temperature.

### 2.4. Mass spectrometric conditions

SSI is a novel ionization interface, which generates charged droplets by nitrogen gas flow at the speed of sound [26,27]. An eluent solution is pumped into a SSI chamber through a fused-silica capillary (0.1 mm I.D., 0.2 mm O.D.). The end of the fused-silica capillary extends about 0.5 mm beyond an orifice (0.4 mm I.D.) of the ion source. Nitrogen gas flows through the orifice into the atmosphere and a spray is thus generated in which charged droplets and ions are produced. The generated ions are passed through a first aperture and a second aperture into the mass analyzing region. The main parameters that influenced the signal intensity were the nitrogen gas pressure, a drift voltage between the first aperture and the second aperture, and a first aperture temperature. The optimum conditions were achieved with 300 kPa of nitrogen gas pressure, 100 V of a drift voltage, 150°C of a first aperture temperature. Mass analysis was performed in the negative ion mode. The mass spectrometer was operated using a mass scan range of 500–1200, three microscans with an accumulation time of 500 ms and a photomultiplier voltage of 500 V.

### 2.5. Sample preparation

The toxin-free scallop was treated by the following modified procedure of Lee et al. [28], to evaluate the degree of the signal suppression by coeluting substances.

A 1-g amount of hepatopancreas of a scallop was homogenized with 4 ml of methanol–water (80:20, v/v). After centrifugation, 3 ml of 8% NaCl solution in water was added to the supernatant which was extracted with chloroform (2×5 ml) and evaporated to dryness under nitrogen gas. The residue was redissolved in 1 ml of methanol. This solution was applied in the following experiments.

## 3. Results and discussion

### 3.1. Optimization of SSI

The mass spectra of each compound were measured in the positive and the negative ion modes. All four compounds were detected sensitively in the negative ion mode. SSI produced a soft ionization giving little fragmentation [26,27] and the $[M-H]^-$ ions yielded the most abundant signal for OA, DTX1 and PTX6 and the $[M-2Na+H]^-$ ion for YTX in this study.

Nitrogen gas pressure, a drift voltage and a first aperture temperature were optimized by monitoring the ions, $[M-H]^-$ for OA, DTX1 and PTX6 and $[M-2Na+H]^-$ for YTX by injection of the calibration standards repeatedly.

The nitrogen gas pressure was an important parameter for the formation of charged droplets and its optimum condition depended mainly on eluent flow-rate. In this study, maximum signal intensities were found at 300 kPa of nitrogen gas pressure at 0.2 ml/min of eluent flow-rate. The drift voltage was altered between 30 and 130 V. As a result, the maximum signal intensity was found at 70 V of the drift voltage.

### 3.2. Signal suppression by coeluting substances

To investigate the influences on the MS responses by coeluting substances originated from scallop extracts, the DSPs standard solution was spiked into this extract solution of scallops. In this experiment, 50 μl of the DSPs standard solutions of 100, 400 μg/l and 1 mg/l were added to 50 μl each of the

extract solution. These DSPs spiked into the toxin-free extract solution were analyzed and the results were compared with that of the standard solutions in methanol (Fig. 2). Both the calibration curves of DSPs in the standard solutions in methanol and in the toxin-free extract solution were linear in the concentration range up to 500 μg/l and with a zero intercept to within experimental error. However, the values of DSPs added to the extract solutions were about 19–42% lower than the values of DSPs in the standard solutions in methanol. The rates of signal suppressions were constant and independent of the concentration of DSP in the solutions.

The results show that coeluting substances cause signal suppressions because of the degradation of ionization efficiency and the external standard method is not available for the quantitation of DSPs in the scallops. The following methods to improve the accuracy of quantitation will be considered.

(a) Complete removal of coeluting substances by sample clean-up, e.g. column switching, solid-phase extraction etc.; in this study, a further sample clean-up method was examined by extracting with hexane (2×5 ml) before extraction with chloroform to remove lipids in the scallops. However, any improvements of signal suppression were not observed. Matuszewski et al. [14] point out that coeluting, undetected matrix components may reduce the ion intensity of the analytes; the signal suppressions of the analytes can be improved by selective extraction and improved chromatographic separation in the case of determination of drugs in human plasma. However, in the case of scallops, the matrices of scallops are complex and different in composition from scallop to scallop. Consequently, even if the same extraction procedure is used for each scallop, the extract solution may vary between scallops. This means that the degree of signal suppressions by coeluting substances also varies from scallop to scallop. It is concluded that it will be difficult and impractical to remove coeluting substances completely for the reduction of the signal suppressions.

(b) Correction by using internal standards, that is, $^{13}C$ substituted DSPs substances; the use of isotopically labeled substances is useful for the correction of the signal deviation because they have almost the

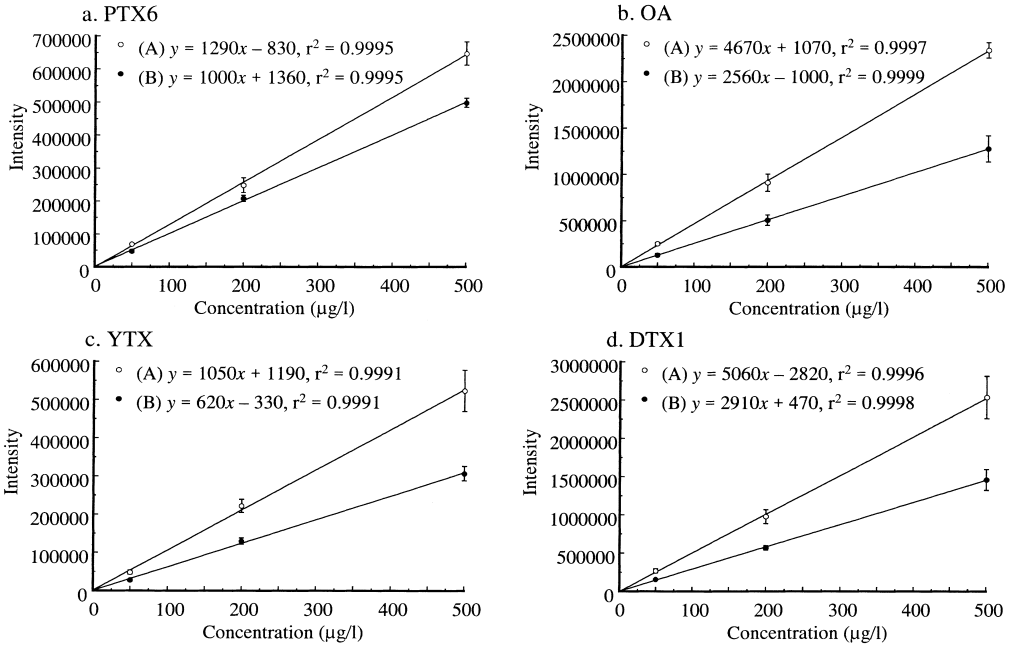


Fig. 2. Calibration curves for DSPs in (A) the standard solutions in methanol (○) and (B) the standard solutions in the poison free scallop extract solution (●) obtained from 10-μl injections onto a 150 mm×2.1 mm I.D. ODS column; LC eluent flow-rate of 0.2 ml/min.

same chemical properties and the same retention times as non-labeled substances. They are generally used in environmental analyses [29]. However, isotopically labeled internal standards may not be available for some analytes due to difficulties associated with synthesis and/or cost [16]. It would be difficult to obtain $^{13}C$-substituted DSPs substances, so this correction method would not be practical.

(c) Preparation of calibration curves by using standardized scallop extracted solutions; a controlled serum is often used for calibration curves in the case of drug analysis in serum by LC–MS [30]. However, standardized scallop extract solutions will not be prepared because matrices vary from scallop to scallop. To evaluate the availability of this correction method, three other scallops were extracted and spiked with the DSPs individually. The concentration of DSPs in the three extract solutions were 200 μg each toxin/l. The three solutions were measured by LC–MS and the deviations of signal suppressions evaluated. The quantitative results are summarized in Fig. 3. All of quantitative values were lower because of signal suppressions by coeluting substances and the degrees of the signal suppression were different from scallop to scallop. This means that matrices in the scallop extract solutions were different from scallop to scallop. As a result, it was impossible to correct the signal suppression by coeluting substances by using another scallop extracts.

(d) Standard addition method, that is, analyzing the extract solution added the known quantity standard solution; the calculation procedure is as follows:

$$X = SI_x/(I_s - I_x)$$

where $X$ is the amount of DSP in the extract solution; $S$ is the amount of DSP spiked into the extract solution; $I_x$ is the signal intensity of DSP in the extract solution and $I_s$ is the signal intensity of DSP in the spiked solution.

This method requires at least two LC–MS runs per analysis — the run of the extract sample and the run of the extract samples spiked with a known quantity of standard DSPs.

Referring to Fig. 2, the calibration curves of DSPs in the extract solution were linear and with a zero intercept to within experimental error. In this report the effectiveness by the single-point standard addition method was examined, which was predicted as the most practical solution. In this correction method, the extract sample is analyzed and then the extract sample with the addition of the calibration standard is analyzed. It was evaluated by using the model sample whether this method was effective for the analysis of DSPs in scallops.

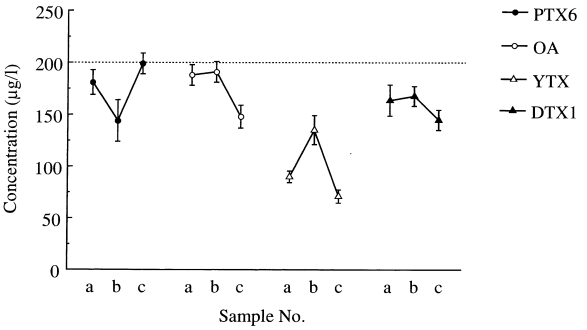


Fig. 3. Quantitative results of four DSPs (200 μg each toxin/l; dashed line) in the three extract solutions (samples a–c). The results were calculated by using the standard solutions in methanol. Each of three samples was measured six times. LC conditions as in Fig. 2.

The model sample was prepared by adding the DSPs standard solutions to the scallop extract. A 50-μl volume of the DSPs standard mixture of 400 μg/l was added to 50 μl of the extract solution. The amounts of each DSP in the model sample were 200 ng each per gram of hepatopancreas, which corresponds to around regulation quantities of DSPs in scallops in Japan. The calibration solution was prepared by spiking the DSPs standard solution into this model sample. The amounts of DSPs spiked were 300 ng each per g of hepatopancreas.

Mass chromatograms of the model sample are shown in Fig. 4. Each peak of DSPs was detected sensitively for the regulation quantities of DSPs in Japan. The quantitative results of the model sample are summarized in Table 1. The quantitative results of the model sample using the external standard by the standard solutions were 15–33% lower than the theoretical values, and this is reasonable considering the results of the preliminary test (see Figs. 2 and 3).

On the other hand, the quantitative results using the standard addition method by the extract solution added with the DSPs standard were in agreement with the theoretical values. The results show that this

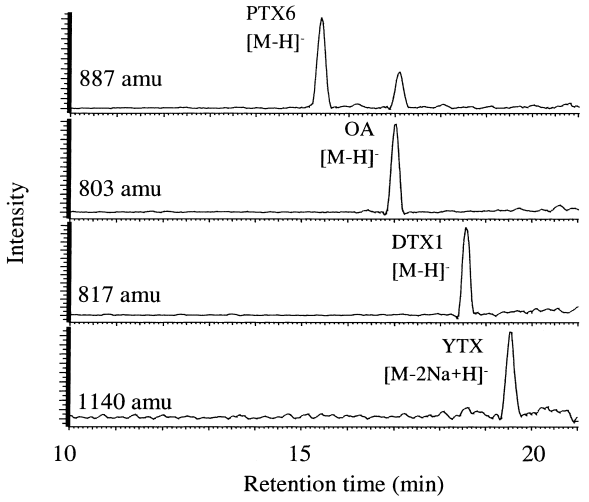


Fig. 4. Mass chromatograms of the model sample (adding the DSPs standard to the toxin-free scallop extract, 2 ng each toxin). LC conditions as in Fig. 2.

method is able to correct the quantities even if coeluting substances from scallops caused the signal suppression. Although this method requires the two LC–MS run per analysis, the correction of quantitative errors was found effective for the four DSPs.

### 3.3. Analytical performance data

Determination of detection limits ($S/N=3$) was performed for the mass chromatographic peaks by a 10-μl injection of the DSP containing standard solution (20 μg each toxin/l) in methanol. Table 2 shows the detection limits of the DSPs with the ions used for detection and calculation. The detection limits of the DSPs were 3 μg/l for OA and DTX1 and 6 μg/l for PTX6 and YTX, therefore the detection limits in injection amounts were in the range 30–60 pg. In the case of the scallop extracts, however, the signal intensity of the DSPs was influenced by coeluting substances originating from the scallops. In the case of the results shown in Fig. 3, for example, signal intensities of PTX6 were reduced maximum 28%, consequently the detection limit of PTX6 was 8.3 μg/l.

The linearity data of the DSPs by LC–MS are shown in Table 3. Linearity of the chromatographic determination was examined for the concentration

Table 2
Detection limits of DSP toxins by LC–MS with SSI interface

| Analyte | Monitored ion | Detection limit[a] (μg/l) |
|---|---|---|
| DTX1 | 817 | 3 (30 pg) |
| OA | 803 | 3 (30 pg) |
| PTX6 | 887 | 6 (60 pg) |
| YTX | 1140 | 6 (60 pg) |

[a] Detection limits ($S/N=3$); injecting 10 μl of the DSP containing standard solution (20 μl each toxin/l) in methanol. LC was performed on to a 150×2.1 mm I.D. ODS column and LC eluent flow-rate of 0.2 ml/min.

Table 3
Linearity data of DSP toxins by LC–MS

| Analyte | Slope±SD[a] | Intercept±SD[a] | Correlation coefficient |
|---|---|---|---|
| DTX1 | 2910±120 | 470±660 | 0.9996 |
| OA | 2560±60 | −1010±860 | 0.9993 |
| PTX6 | 1000±30 | 1780±370 | 0.9995 |
| YTX | 620±20 | 330±280 | 0.9994 |

The linearity data were calculated at concentrations from 20 to 500 μl/l, injecting 10 μl of the DSPs standard added in the poison free extract solution. LC conditions as in Table 2.

[a] $n=6$.

Table 1
Quantitative results of the model sample by using the standard addition method and the external standard method by the standard solutions

| Calibration method | Amount±SD[c] (ng/g)[d] | | | |
|---|---|---|---|---|
| | PTX6 | OA | YTX | DTX1 |
| Model sample (theoretical) | 200 | 200 | 200 | 200 |
| External standard method[a] | 170±8 | 134±14 | 135±8 | 138±6 |
| Standard addition method[b] | 197±9 | 213±20 | 215±12 | 214±10 |

[a] The calibration curves were prepared using DSPs standard solutions in methanol.

[b] The calculation was carried out by adding the DSPs standard solution to the model sample.

[c] $n=6$.

[d] ng/g hepatopancrea extract.

Table 4
Intra-day accuracy and precision data for LC–MS by using the standard addition method

| | Compound | | | |
|---|---|---|---|---|
| | PTX6 | OA | YTX | DTX1 |
| Concentration (μg/l) | 200 | 200 | 200 | 200 |
| Mean±SD[a] (μg/l) | 197±9 | 213±20 | 215±12 | 214±10 |
| RSD (%) | 4.4 | 9.4 | 5.6 | 4.5 |
| Bias (%) | −1.5 | 6.5 | 7.5 | 7.0 |

[a] $n=6$.

range 20–500 μg/l in the scallop extract solutions. Linearity was good for all DSPs (correlation coefficient, $r^2>0.999$). The slopes of the calibration curve were between 620 and 2560 response units because ionization efficiencies of the DSPs were different. Intercepts of the calibration curve were between −1010 to 1780 response units, which were smaller than signal responses equivalent to the detection limits of DSPs.

Intra-day statistics for accuracy and precision by using the standard addition method are shown in Table 4. The accuracy of the standard addition method, expressed in terms of bias (deviation from the true values) was between −1.5 and 7.5% for the concentration of 200 μg each toxin/l. The precision, given by relative standard deviations, was between 4.4 and 9.4%.

## 4. Conclusion

LC–MS is suitable for analysis of DSPs because of high selectivity and sensitivity. However, it is clear that the suppression of the ionization efficiency occurs due to coeluting substances and causes variation of responses of LC–MS and makes it difficult to quantify the DSPs by using the pure standard solutions. The standard addition method was examined for the correction of the signal suppression, that is, the method to quantify the DSPs by calibration with the DSPs solution added in the extract solution. This method is practical in the case of analysis of DSPs in scallops, although it requires two LC–MS run per analysis. The quantitative results of DSPs in scallops extracts by external standards were 15–33% lower than the theoretical values because of the signal suppression. On the other hand, the quantitative results of DSPs by this method were good agreement with the theoretical values. The accuracy and precision were good in the concentration range relevant to the regulation quantities of DSPs in scallops in Japan.

## Acknowledgements

This study was supported mainly with funding from the Japan Fisheries Agency through the Marino-Forum 21. The authors wish to thank Dr. T. Yasumoto (Japan Food Research Laboratories) and his colleagues for valuable discussions.

## References

[1] B. Luckas, J. Chromatogr. 624 (1992) 439.
[2] T. Yasumoto, M. Fukui, K. Satake, K. Sugiyama, J. AOAC Int. 78 (1995) 574.
[3] C. Hummert, J.L. Shen, B. Luckas, J. Chromatogr. A 729 (1996) 387.
[4] K. Akasaka, H. Ohrui, H. Meguro, T. Yasumoto, J. Chromatgr. A 729 (1996) 381.
[5] S.L. Morton, D.R. Tindall, Toxicon 34 (1996) 947.
[6] M. Comesana-Losada, A. Gago-Martinez, J.A. Rodrigues-Vazquez, Analyst 121 (1996) 1665.
[7] S.S. Kelly, A.G. Bishop, E.P. Carmody, K.J. James, J. Chromatgr. A 749 (1996) 33.
[8] T. Yasumoto, A. Takizawa, Biosci. Biotech. Biochem. 61 (1997) 1775.
[9] B. Aase, A. Rogstad, J. Chromatgr. A 764 (1997) 223.
[10] P. Ciminiello, E. Fattorusso, M. Forino, S. Magno, R. Poretti, M. Satake, R. Viviani, T. Yasumoto, Toxicon 35 (1997) 177.
[11] J.C. Gonzalez, M.R. Vieytes, J.M. Vieytes, L.M. Botana, J. Chromatogr. A 793 (1998) 63.
[12] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972A.
[13] U.A. Mirza, B.T. Chait, Anal. Chem. 66 (1994) 2898.
[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[15] R. Swart, P. Koivisto, K.E. Markides, J. Chromatogr. B 736 (1999) 247.
[16] B.K. Choi, A.I. Gusev, D.M. Hercules, Anal. Chem. 71 (1999) 4107.
[17] K.J. James, A.G. Bishop, R. Draisci, L. Palleschi, C. Marchiafava, E. Ferretti, M. Satake, T. Yasumoto, J. Chromatogr. A 844 (1999) 53.
[18] R. Draisci, L. Palleschi, L. Giannetti, L. Lucentini, K.J. James, A.G. Bishop, M. Satake, T. Yasumoto, J. Chromatogr. A 847 (1999) 213.
[19] S.J. Locke, P. Thibault, Anal. Chem. 66 (1994) 3436.

[20] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, A. Stacchini, Toxicon 33 (1995) 1591.
[21] M.A. Quilliam, J. AOAC Int. 78 (1995) 555.
[22] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, K.J. James, A. Furey, M. Gillman, S.S. Kelly, J. AOAC Int. 81 (1998) 441.
[23] R. Draisci, L. Giannetti, L. Lucentini, E. Ferretti, L. Palleschi, C. Marchiafava, Rapid Commun. Mass Spectrom. 12 (1998) 1291.
[24] S. Pleasance, M.A. Quilliam, J.C. Marr, Rapid Commun. Mass Spectrom. 6 (1992) 121.
[25] J.L. Shen, C. Hummert, B. Luckas, Fresenius J. Anal. Chem. 357 (1997) 101.
[26] A. Hirabayashi, M. Sakairi, H. Koizumi, Anal. Chem. 66 (1994) 4557.
[27] A. Hirabayashi, M. Sakairi, H. Koizumi, Anal. Chem. 67 (1995) 2878.
[28] J.S. Lee, T. Yanagi, R. Kenma, T. Yasumoto, Agric. Biol. Chem. 51 (1987) 877.
[29] J.W. Hills, H.H. Hill Jr., J. Chromatogr. A 679 (1994) 319.
[30] J. Darius, P. Banditt, J. Chromatogr. B 738 (2000) 437.