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Improved Solid-Phase Extraction Procedure in the Analysis of Paralytic Shellfish Poisoning Toxins by Liquid Chromatography with Fluorescence Detection

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The analysis of shellfish extracts for the determination of paralytic shellfish poisoning (PSP) toxins by liquid chromatography with fluorescence detection repeatedly showed the presence of a compound suspected to interfere with gonyautoxin 4. The first aim of this study was to confirm by liquid chromatography coupled to tandem mass spectrometry that this compound was not gonyautoxin 4. The second part of this work was to improve a nonvolumetric C_{18} solid-phase extraction (SPE) procedure to evaluate the removal of the interference associated with the recovery of PSP toxins. The cleanup procedure was modified into a volumetric SPE procedure and proved to efficiently and totally remove the interference while recovering from 78 to 85% of the PSP toxins available as commercial standards (saxitoxin, neosaxitoxin, gonyautoxins 1−4) and considered as major PSP toxins in human intoxication, with 85% recovery for gonyautoxin 4. The efficiency of this cleanup procedure was checked on shellfish extracts containing this interference and originating from France and Turkey.

KEYWORDS: Shellfish; paralytic shellfish poisoning toxins; gonyautoxin 4 interference; C_{18} cleanup; liquid chromatography; fluorescence detection; mass spectrometry

INTRODUCTION

The paralytic shellfish poisoning (PSP) toxins (**Figure 1**) are potent neurotoxins active on voltage-gated sodium channels of excitable cells (*1*). This family includes more than 20 related compounds (*2*, *3*) mainly produced by toxigenic dinoflagellates in marine environment (*4*). The accumulation of these toxins in filter-feeding shellfish consumed by humans may induce an intoxication known as paralytic shellfish poisoning (*5*). To protect consumers from this syndrome, monitoring programs were implemented in many countries to avoid the marketing of unsafe shellfish, in compliance with European Directive 91/492 EEC. This specifies that the total PSP toxin content in shellfish determined by the biological testing method must not exceed 80 μg/100 g of meat. In France, this is performed according to a validated mouse bioassay (*6*), recommended within the European Union by the Community Reference Laboratory on Marine Biotoxins as the official biological method (*7*). Although the mouse bioassay enables the determination of the overall toxicity of shellfish by injecting an extract into mice, it does not give any information on the panel of toxins present. This can be achieved only by separative techniques, the major one in use being liquid chromatography with fluorescence detection (LC-FLD) (*8*−*10*).

However, LC-FLD analyses can be misleading as several studies reported the presence of fluorescent compounds potentially interfering with some of the PSP toxins (*11*−*15*) and particularly with gonyautoxin 4. In this case, because the LC-FLD technique can be used also as a quantitative tool, total PSP toxin content could be overestimated, generating discrepancies with global toxin content estimated by the mouse bioassay. To overcome this interference problem, solid-phase extraction (SPE) procedures were developed to clean up the shellfish extracts prior to chromatographic or electrophoretic analysis, using reversed-phase cartridges (C_{18}) only (*9*, *16*, *17*) or in association with ion-exchange cartridges (*10*, *18*). Nevertheless, studies reporting the use of SPE procedures emphasized either the removal of the interference or the recovery of the PSP toxins, but not both aspects, which are important for quantification. Thus, Oshima (*9*) developed a nonvolumetric SPE procedure to remove an interference eluting near gonyautoxin 4 and gonyautoxin 6, but information regarding the recovery of the PSP toxins was lacking. Leao et al. (*13*) studied the recovery of saxitoxin and decarbamoylsaxitoxin in a comparison of three of these C_{18} cleanup procedures, including the SPE procedure of Oshima (*9*), but this study was not focused on the removal of potential interferences.

In our laboratory, LC-FLD analyses of shellfish extracts from different origins (France and Turkey) repeatedly showed the presence of a compound with the same retention time as gonyautoxin 4. However, we suspected this compound to be

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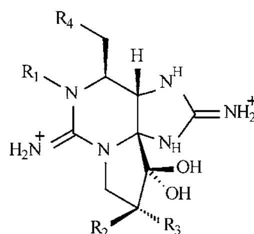

	R1	R2	R3	R4
Saxitoxin	H	H	H	$OCONH_2$
Decarbamoylsaxitoxin	H	H	H	OH
Gonyautoxin-2	H	H	OSO_3^-	$OCONH_2$
Gonyautoxin-3	H	OSO_3^-	H	$OCONH_2$
Gonyautoxin-5	H	H	H	$OCONHSO_3^-$
C1	H	H	OSO_3^-	$OCONHSO_3^-$
C2	H	OSO_3^-	H	$OCONHSO_3^-$
Neosaxitoxin	OH	H	H	$OCONH_2$
Gonyautoxin-1	OH	H	OSO_3^-	$OCONH_2$
Gonyautoxin-4	OH	OSO_3^-	H	$OCONH_2$
Gonyautoxin-6	OH	H	H	$OCONHSO_3^-$
C3	OH	H	OSO_3^-	$OCONHSO_3^-$
C4	OH	OSO_3^-	H	$OCONHSO_3^-$

Figure 1. Structures of the PSP toxins.

an interference because of the absence of toxicity to mice of the extracts and the absence of gonyautoxin 1, which is always associated with its epimer, gonyautoxin 4. The purposes of this study were, first, to confirm that the suspect compound was not gonyautoxin 4, by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and, second, to improve a SPE procedure described in the literature (*9*, *13*) to specifically remove the gonyautoxin 4 interference while ensuring a high recovery rate of the PSP toxins available as commercial standards (saxitoxin, neosaxitoxin, and gonyautoxins 1−4), all considered as major PSP toxins in human intoxication.

MATERIALS AND METHODS

Toxin Standards. A set of four calibration solutions of PSP toxins (saxitoxin, neosaxitoxin, gonyautoxin 2/gonyautoxin 3, and gonyautoxin 1/gonyautoxin 4, respectively) was purchased from the Institute for Marine Biosciences, National Research Council Canada, Halifax, Canada. A mixture of these six PSP toxins was prepared by diluting aliquots of the calibration solutions in 0.1 M acetic acid to reach individual toxin concentrations of 0.40 μg/mL for saxitoxin, neosaxitoxin, gonyautoxin 1, and gonyautoxin 2, 0.10 μg/mL for gonyautoxin 3, and 0.18 μg/mL for gonyautoxin 4.

Shellfish Extracts. The four extracts used in this study were obtained by boiling homogenized shellfish tissue for 5 min with 0.1 N HCl, as described in the AOAC procedure (*6*). All four extracts showed the presence of a gonyautoxin 4 interference and the absence at a detectable level of gonyautoxin 1, the epimer concomitantly reported with gonyautoxin 4, as determined by the LC-FLD method of Oshima (*9*). Two extracts were prepared from shellfish harvested in France and were kindly provided by the Laboratoire Vétérinaire Départemental des Bouches du Rhône, France (extracts FRA01 and FRA02). These extracts were negative with the PSP mouse bioassay (detection limit of 40 μg of saxitoxin equivalent per 100 g of meat) and contained traces of saxitoxin and a high amount of gonyautoxin 4 interference. The other two extracts were prepared from shellfish harvested in Turkey and kindly provided by Dr. Sibel Dolen from the Institute of Marine Sciences and Technology, Izmir University, Turkey (extracts TUR01 and TUR02). These extracts were negative with the PSP mouse bioassay and contained only the gonyautoxin 4 interference in high amount, with no trace of any of the six mentioned PSP toxins.

Improvement of the Cleanup Procedure. We decided to adapt the cleanup procedure described by Oshima (*9*) to enable the removal of the gonyautoxin 4 interference while ensuring a high recovery rate of the six tested PSP toxins, and the first step was to follow the elution patterns of the PSP toxins and the interference on the C_{18} cartridge. After conditioning the Sep-Pak C_{18} Plus cartridge (Waters, Saint-Quentin en Yvelines, France) with 6 mL of methanol and 6 mL of distilled water, we loaded a 3-mL aliquot of the mixture of PSP toxins onto the cartridge, and the elution was carried out with 5 mL of distilled water. The cartridge effluent was collected as successive 0.5-mL fractions, every fraction being individually analyzed by LC-FLD. The same procedure was applied to the shellfish extract FRA01 to determine the elution pattern of the interference.

Cleanup of Shellfish Extracts Using the Improved Procedure. The following cleanup procedure was applied to the shellfish extracts prior to LC-FLD analysis: a C_{18} cartridge was conditioned with 6 mL of methanol and 6 mL of distilled water, then a 3-mL aliquot of shellfish extract was loaded onto the cartridge, and the elution was carried out with 1 mL of distilled water to collect accurately 4 mL of cartridge effluent in a graduated conical tube. This 4-mL fraction was likely to contain only the PSP toxins, whereas the interference could be partially recovered from a second 4-mL fraction, collected separately, after elution with another 4 mL of distilled water. The efficiency of this SPE procedure to totally remove the interference as well as to recover the PSP toxins was verified by cleaning up the four crude shellfish extracts (FRA01, FRA02, TUR01, and TUR02) and the shellfish extract TUR01 spiked with the PSP toxins.

Liquid Chromatography with Fluorescence Detection. The post-column LC-FLD analysis was performed according to the method of Oshima (*9*) with the following minor modifications: an HP1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) was used to deliver a mobile phase of 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) for the gonyautoxin group (gonyautoxins 1−4) and 2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer (pH 7.1) containing 4.8% (v/v) of acetonitrile for the saxitoxin group (saxitoxin and neosaxitoxin). The flow rate was set to 0.8 mL min^{-1}, and the separation of analytes was performed on a 250 × 4.6 mm i.d., 5 μm, Develosil C_8 reversed-phase column (Interchim, Montluçon, France), heated at 22 °C. Whatever the mobile phase, the eluate from the column was continuously oxidized with 7 mM periodic acid in 50 mM potassium phosphate buffer (pH 9.0; flow rate = 0.4 mL min^{-1}) before passing through a 10-m Teflon tubing coil (0.5 mm i.d.) immersed in a 50 °C water bath. The eluate was then continuously acidified with 0.5 M acetic acid (flow rate = 0.4 mL min^{-1}), and the toxins were finally detected by a fluorescence detector (λ_{ex} = 330 nm and λ_{em} = 390 nm). Data acquisition and processing was performed with the HP-ChemStation software. The C toxins were not searched for because standards were not available at the time of the study. PSP toxin concentrations were determined by means of a calibration curve, and the amount of interference was expressed as the peak area.

Liquid Chromatography Coupled with Tandem Mass Spectrometry. LC-MS/MS analyses were performed according to the method described by Lagos et al. (*19*) with the following slight modifications: an HP1100 series liquid chromatograph (Agilent Technologies) was used to deliver a mobile phase of 10 mM heptafluorobutyric acid/acetonitrile (95:5, v/v) and the flow rate was set to 50 μL min^{-1}. Separation of analytes was performed on a 150 × 2.0 mm i.d., 5 μm, Uptisphere ODB C_{18} reversed-phase column (Interchim). An API 2000 triple-quadrupole mass spectrometer (PE-Sciex, Les Ulis, France) equipped with an atmospheric pressure ion source was used as detector. Compressed air served both as sheath gas and as auxiliary gas at an operating pressure of 15 and 55 psi, respectively. High-purity nitrogen was used as curtain gas at 12 psi. Electrospray ionization was carried

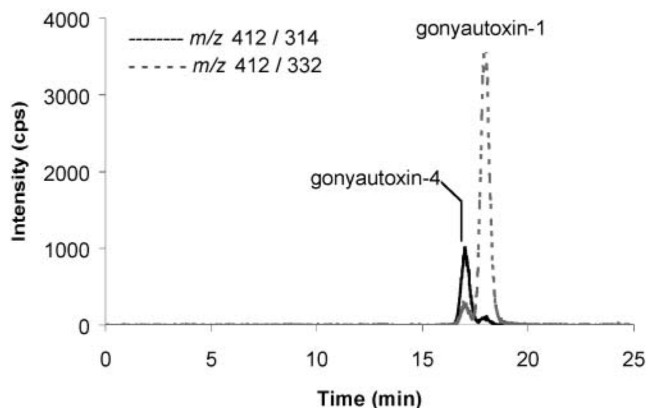

Figure 2. LC-MS/MS chromatogram of a standard mixture containing the epimers gonyautoxin 1 and gonyautoxin 4.

out with a spray voltage of +5.5 kV, and the auxiliary gas was heated at 400 °C. Analyst software (PE-Sciex, Concord, ON, Canada) was used for instrumental control, data acquisition, and data processing. All spectra were acquired in positive ion mode. The transitions *m/z* 412/314 and *m/z* 412/332 were monitored in multiple reaction monitoring mode to detect the gonyautoxin 4 ($[M + H]^+$ *m/z* 412).

RESULTS AND DISCUSSION

LC-MS/MS analyses confirmed that the compound present in the four shellfish extracts was not gonyautoxin 4 but an interference. **Figure 2** shows the mass chromatogram of a standard mixture of the gonyautoxin 4/gonyautoxin 1 epimers, and none of the transitions *m/z* 412/314 ($[M - H_2O - SO_3 + H]^+$) and *m/z* 412/332 ($[M - SO_3 + H]^+$) specific to both epimers was detected in any of the four analyzed shellfish extracts. The absence, at a detectable level, of gonyautoxin 4 in the shellfish extracts is consistent with the results of the PSP mouse bioassay, indicating that all four extracts were not toxic to mice.

After the confirmation of the presence of an interference with the gonyautoxin 4 in the four shellfish extracts, we proceeded to the improvement of the cleanup procedure originally developed by Oshima (*9*). The elution patterns of the six tested PSP toxins on the C_{18} cartridge were determined from the mixture of PSP toxins and that of the interference was determined in parallel from the crude extract FRA01 as shown in **Figure 3**. All tested PSP toxins have very similar elution patterns. The elution starts after the collection of the first 0.5-mL fraction (approximate holdup volume of the cartridge), and the toxins are nearly quantitatively eluted after the collection of a total volume of 5 mL, with a maximum recovery rate ranging from 91% (gonyautoxin 1) to 97% (saxitoxin). The elution pattern of the interference on the C_{18} cartridge is different because its elution starts after the collection of 4 mL. A significant portion of the interference is trapped in the cartridge as only 20% was eluted after a 8-mL effluent collection.

The recovery rate of saxitoxin using this procedure is ~20% higher than reported in a previous study (*13*). However, the discrepancy in the results is probably due to the fact that Leao et al. (*13*) took into account both the extraction step and the cleanup procedure in the calculation of the saxitoxin recovery rate, whereas we considered only the cleanup procedure in our study.

A more striking difference with the study performed by Leao et al. (*13*) comes from the volume collected from the cartridge to ensure a satisfactory recovery of the toxins. Whereas a 3.5-mL fraction has to be collected according to the procedure

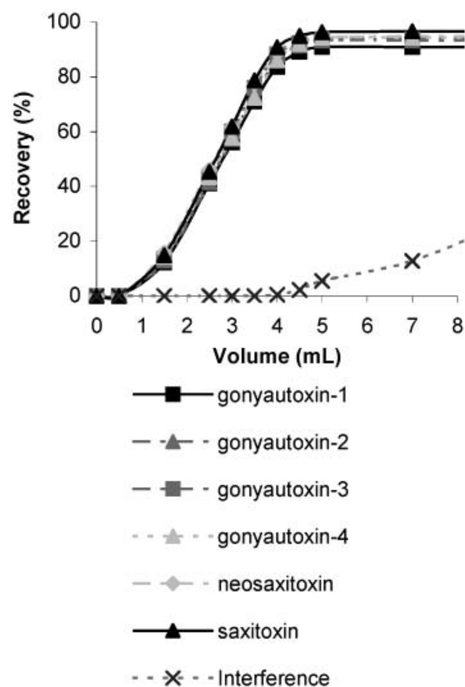

Figure 3. Elution patterns of the six tested PSP toxins and the interference on a C_{18} cartridge determined with the mixture of PSP toxins and the shellfish extract FRA01, respectively.

described herein to recover ~75% of saxitoxin (**Figure 3**), Leao et al. (*13*) reported that only 1.5−2.0 mL of effluent was sufficient to ensure the same saxitoxin recovery when performing the SPE procedure of Oshima (*9*). The first explanation for this difference probably comes from the fact that the SPE procedure used by Leao et al. (*13*) is nonvolumetric; therefore, the calculation of the PSP recovery rate cannot be precise and is prone to important variations. Because the same type of C_{18} cartridge was used in both studies, this is unlikely to account for the difference in the saxitoxin recovery, unless the cartridge lots were heterogeneous in terms of separation efficiency. A major difference in the experimental design of both studies comes from the nature of the solution purified on the C_{18} cartridge, a shellfish extract prepared from mussels spiked with PSP toxins being used by Leao et al. (*13*), versus a mixture of PSP toxins in our study. The possibility of a matrix effect being responsible for the discrepancy in the results was ruled out as we obtained similar PSP toxin recoveries after the cleanup of the mixture of PSP toxins and the extract TUR01 spiked with PSP toxins. Another difference in the experimental design of both studies comes from the nature and the concentration of the acid used for toxin extraction (0.1 N hydrochloric acid in our study versus 0.2 M acetic acid), and Leao et al. (*13*) reported that the concentration of the acid used is critical for both the extraction and cleanup of shellfish extracts. The discrepancy with the study of Leao et al. (*13*) in the elution pattern of saxitoxin underscores the importance of testing a cleanup procedure prior to its use in routine analysis; otherwise, we would have only recovered 15−30% of PSP toxins by applying the original SPE procedure described by Oshima (*9*).

Considering the elution patterns of the PSP toxins and the interference, we decided for the cleanup of the shellfish extracts on a routine basis to collect the first 4-mL fraction to ensure the interference removal and to recover 84−91% of the PSP toxins (**Figure 3**). The efficiency of this procedure was first checked by cleaning up the shellfish extract TUR01 spiked with the PSP toxins (**Figure 4**). Average toxin recovery rates ranging

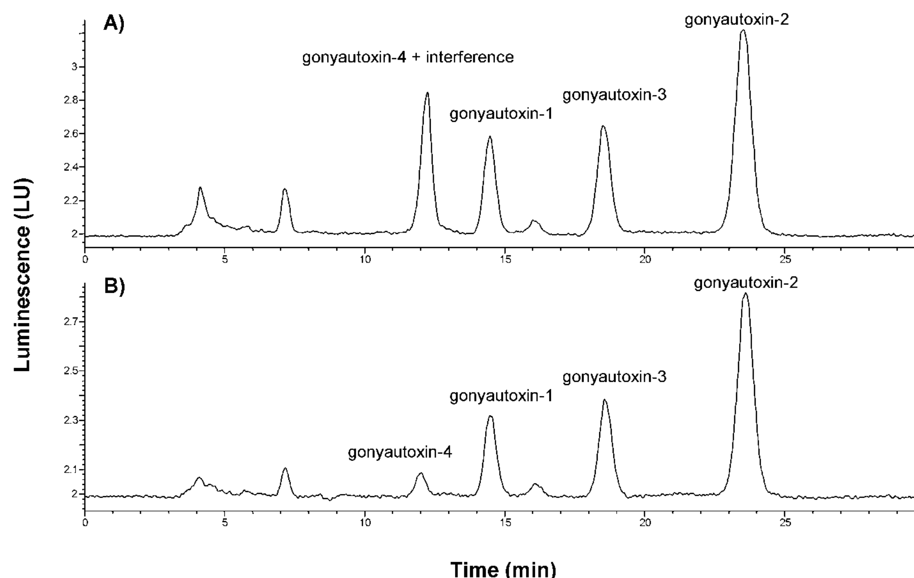

Figure 4. LC-FLD chromatograms corresponding to the shellfish extract TUR01 spiked with PSP toxins, (A) without cleanup and (B) with cleanup, on a C_{18} cartridge (first 4-mL fraction).

Table 1. Average Recovery of the PSP Toxins after Cleanup of the Spiked Shellfish Extract TUR01 and Collection of the First and Second 4-mL Fractions, Respectively

	av recovery (RSD; $n = 3$), %		
	1st 4-mL fraction	2nd 4-mL fraction	total
gonyautoxin 1	78 (9)	16 (6)	94
gonyautoxin 2	83 (5)	14 (14)	97
gonyautoxin 3	78 (2)	16 (4)	94
gonyautoxin 4	85 (7)	nd[a]	>85
saxitoxin	85 (4)	16 (14)	101
neosaxitoxin	83 (5)	14 (13)	97

[a] Not determined because the gonyautoxin 4 and the interference coeluted.

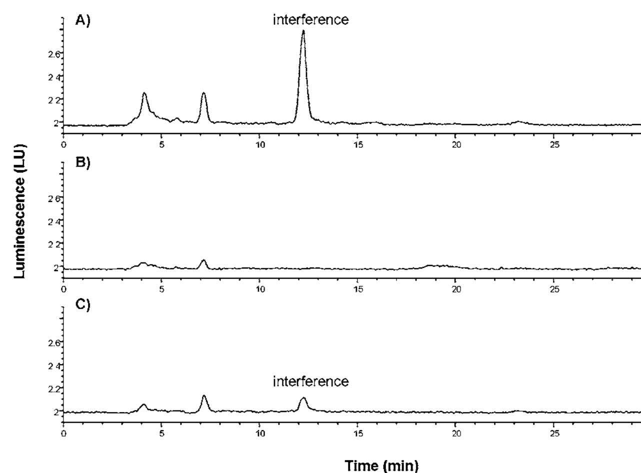

Figure 5. LC-FLD chromatograms corresponding to the shellfish extract TUR01, (A) without cleanup and (B, C) with cleanup on a C_{18} cartridge, after collection of (B) the first 4-mL fraction and (C) the second 4-mL fraction.

from 78% (gonyautoxin 1) to 85% (saxitoxin and gonyautoxin 4) (**Table 1**) are in agreement with those of the mixture of PSP toxins (**Figure 3**). However, this did not confirm the complete removal of the interference from the first 4-mL fraction. Therefore, the cleanup efficiency was further checked by applying this procedure to the four crude shellfish extracts. The typical LC-FLD chromatograms corresponding to the first and second 4-mL fractions collected from the C_{18} cartridge after cleaning up any of the four shellfish extracts are presented in **Figure 5**. This confirmed that the cleanup procedure successfully removed the interference, as the latter did not elute in the first 4-mL fraction, which was shown to contain the major amounts of the PSP toxins and particularly gonyautoxin 4.

As the recovery of the PSP toxins following the SPE procedure is not 100% but between 78 and 85%, a correction factor (CF) should be applied to compensate for the slight decrease in concentration resulting from the dilution. In theory, this CF should be 1.3 as 3 mL of extract was loaded onto the cartridge for a final volume of 4 mL, and, in practice, the CF values for the different toxins are close to this value as they range from 1.2 (saxitoxin and gonyautoxin 4) to 1.3 (gonyautoxin 1).

The occurrence of this gonyautoxin 4 interference is not systematic as it was found solely in a few shellfish extracts originating from France and Turkey. Furthermore, this interference phenomenon is not unique and exceptional because some other studies also reported the presence of naturally fluorescent compounds potentially interfering with some of the PSP toxins (*11*−*15*).

The nature of the gonyautoxin 4 interference present in our shellfish extracts is still unknown, but one hypothesis is that it might be a fluorescent compound synthesized by bacteria that are symbionts of PSP-producing dinoflagellates. Indeed, Baker et al. (*15*) reported the presence of five bacterial compounds interfering with the gonyautoxin 4. There is also in the literature an account of the production of a neosaxitoxin imposter by a bacterial strain (*14*). The hypothesis of a bacterial origin for the compounds interfering with the PSP toxins is possible but not necessarily systematic, and each case should be individually investigated to elucidate the nature of the interfering compound.

The cleanup procedure described herein and adapted from Oshima (*9*) enables both the removal of the gonyautoxin 4 interference and a satisfactory recovery rate of the major PSP toxins (78−85%), with 85% recovery for gonyautoxin 4. Furthermore, a nonvolumetric procedure was transformed into a volumetric one, which is more suitable as it takes into account the dilution due notably to the holdup volume of the cartridge.

Our study also emphasizes the necessity to apply a cleanup step prior to LC-FLD analysis. Indeed, complex matrices such as shellfish extracts are prone to contain many possible interfering compounds that are likely to hinder the detection of the PSP toxins, such as an interfering compound mistaken for a PSP toxin or a PSP toxin hidden by an interference, and must therefore be removed from the analyzed extracts. Indeed, there are many accounts in the literature of fluorescent compounds mistaken for PSP toxins. Furthermore, it is necessary to test a reported SPE procedure when a specific application is required. This modified SPE procedure enables the use of LC-FLD as a reliable tool for both identification and quantification of PSP toxins detected by the official mouse bioassay.

ABBREVIATIONS USED

SPE, solid-phase extraction; PSP, paralytic shellfish poisoning; LC-FLD, liquid chromatography with fluorescence detection; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; CF, correction factor.

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