UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE VETERINARIA

DEPARTAMENTO DE FARMACOLOGÍA

DESARROLLO DE TÉCNICAS DE IDENTIFICACIÓN, DETECCIÓN Y PURIFICACIÓN DE TOXINAS LIPOFÍLICAS

Tesis doctoral

Mª Paz Otero Fuertes

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INFORMA

Que la tesis doctoral titulada "Desarrollo de técnicas de identificación, detección y purificación de toxinas lipofílicas", recogida en la presente memoria, de la que es autora la Licenciada en Ciencia y Tecnología de los Alimentos por la Universidad de Santiago de Compostela, Mª Paz Otero Fuertes, ha sido realizado bajo su codirección y cumple las condiciones exigidas para que su autora pueda optar al grado de Doctora por la Universidad de Santiago de Compostela, otorgando su aprobación para la lectura y defensa de la misma.

Para que así conste a los efectos oportunos, firma la presente en Lugo, a 5 de Diciembre de 2012.

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Abreviaturas

ACN:	acetonitrilo.
ASP:	intoxicación amnésica por consumo de molusco.
AZAs:	azaspirácidos.
BTXs:	brevetoxinas.
Cs:	N-sulfocarbamoil-11-hidroxisulfatotoxinas.
CFP:	intoxicación ciguatérica por consumo de pescado.
CIs:	iminas cíclicas.
CTXs:	ciguatoxinas.
C-CTXs:	ciguatoxinas del Caribe.
DA:	ácido domoico.
DSP:	intoxicación diarreica por consumo de molusco.
DTXs:	dinofisistoxinas.
FLD:	fluorescencia.
FP:	polarización de la fluorescencia.
GTXs:	gonyautoxinas
GYMs:	gimnodiminas.
HABs:	floraciones de algas nocivas.
HPLC:	cromatografía líquida de alta eficacia.
HPLC-UV:	HPLC con detección ultravioleta.
HPLC-FLD:	HPLC con detección de fluorescencia.
I-CTXs:	ciguatoxinas del Índico.
i.p.:	intraperitoneal.
LC-MS/MS:	cromatografía liquida con detección por espectrometría de masas.
LD_{50}:	dosis letal 50%.
LD_{100}:	dosis letal 100%.
LD_{99}:	dosis letal 99%.
LOD:	límite de detección.
LOQ:	límite de cuantificación.
mAChR:	receptores muscarínicos de acetilcolina.

MBA:	bioensayo en ratón.
MRM:	monitoreo de reacciones múltiples.
MS:	espectrometría de masas.
MS/MS:	tándem espectrometría de masas.
MTXs:	maitotoxinas.
nAChR:	receptores nicotínicos de acetilcolina.
NSP:	intoxicación neurotóxica por consumo de molusco.
OA:	ácido okadaico.
P-CTXs:	ciguatoxinas del Pacífico.
PnTXs:	pinnatoxinas.
PSP:	intoxicación paralizante por consumo de molusco.
PtTXs:	pteriatoxinas.
PlTXs:	palitoxinas.
PTXs:	pectenotoxinas.
RMN:	resonancia magnética nuclear.
SPXs:	espirólidos.
STXs:	saxitoxinas.
TTXs:	tetrodotoxinas.
UV:	ultravioleta.
UPLC-MS/MS:	cromatografía liquida de ultra eficiencia con detección por espectrometría de masas.
UE:	Unión Europea.
YTXs:	yessotoxinas.
13-desMeC:	13-desmetil espirólido C.
13-desMeD:	13-desmetil espirólido D.
13,19-didesMeC :	13,19-didesmetil espirólido C.
20-MeG:	20-metil-espirólido G.
27-OH-13,19-didesMeC:	7-hidroxi-13,19-didesmetil espirólido C.

Índice

1. Introducción

1. 1. Toxinas Marinas

1.1.1. Generalidades

Las ficotoxinas son productos naturales sintetizados generalmente por microorganismos unicelulares pertenecientes al grupo de los dinoflagelados aunque ciertas diatomeas, cianobacterias y probablemente algunas bacterias están también implicadas en la producción de toxinas que afectan gravemente a la salud humana [1]. Estas toxinas son responsables de síndromes de intoxicación alimentaria desde latitudes tropicales a polares [2] y causan más de 60.000 incidentes en todo el mundo cada año con una tasa de mortalidad del 1,5% [3]. Además de intoxicaciones humanas, también se han registrado mortandades en aves, focas, leones marinos y ballenas [4-7].

Las proliferaciones de microalgas se deben a que convergen en el medio marino una serie de condiciones óptimas y propicias para un gran desarrollo de estos microorganismos, los cuales en tan solo uno o dos días se reproducen alterando de forma significativa la composición específica del plancton marino y originando las comúnmente mareas rojas, conocidas actualmente por floraciones de algas nocivas, empleándose el término en inglés Harmful Algal Blooms (HABs). Estas floraciones están formadas por varios millones de células por litro y a menudo se presentan en forma de manchas discretas o franjas, ocupando capas bien definidas en la columna de agua. Esta proliferación puede ser de color rojo, violeta, azul, verde o incolora dependiendo de los pigmentos y del organismo productor que predomine. La intensidad de color va a depender de la concentración de organismos y de la profundidad a que se encuentre. Las HABs están directamente relacionados con la temperatura del agua, la luz, la salinidad, la presencia de nutrientes y de otras condiciones ambientales [8]. Si las temperaturas caen por debajo de 4ºC, los dinoflagelados sobreviven en forma de quistes enterrados en las capas superiores de los sedimentos. Fenómenos climáticos inusuales como lluvias abundantes y sequias también pueden ocasionar floraciones así como los cambios climáticos globales que, igualmente, se han relacionado a un incremento en las floraciones de algas [9]. Las HABs pueden ocurrir también debido a la transferencia de algas de unos lugares a otros como sucede con el agua de lastre en los barcos [10]. El que predominen unos organismos sobre otros se debe a las condiciones

particulares de salinidad, temperatura y nutrientes. Determinadas condiciones del agua del mar pueden favorecer la reproducción y desarrollo de ciertos organismos que encuentran, en estas circunstancias, posibilidades para su proliferación [11]. No obstante, tanto los factores genéticos y ambientales que favorecen la existencia de especies de fitoplancton tóxico, como el mecanismo por el cual estos organismos producen toxinas, no están plenamente esclarecidos. Se desconoce la naturaleza precisa de los factores que ponen en marcha un clon tóxico [12]. Hay pocos estudios sobre el efecto de los factores ambientales en la producción de toxinas en las especies de dinoflagelados. La relación entre los factores genéticos y ambientales y la producción de toxinas es compleja entre los dinoflagelados [8].

Los moluscos bivalvos filtradores (mejillones, almejas, berberechos y vieiras) que se han alimentado de dinoflagelados tóxicos retienen la toxina durante periodos de tiempo variables que dependen del tipo de molusco y del compuesto pero no mueren. Algunos eliminan la toxina muy rápidamente y son tóxicos únicamente durante el momento de la proliferación, mientras que otros acumulan la toxina en sus tejidos fundamentalmente en su glándula digestiva o hepatopáncreas durante un largo periodo, incluso un año. De esta forma, actúan como vectores para transferir estos compuestos a peces, pájaros y humanos. La frecuencia y la distribución de las floraciones de algas tóxicas es cada vez mayor y constituye un gran problema económico, ambiental y de salud pública mundial.

Las toxinas marinas comprenden un amplio espectro de sustancias con estructura molecular, mecanismo de acción y actividad biológica muy diversa. Proporcionan una gran variedad de compuestos con estructuras moleculares novedosas susceptibles de ser sintetizadas o manipuladas para la obtención de productos útiles desde un punto de vista farmacológico. Su clasificación ha sido abordada de varias formas: según su similitud estructural [1], en base a su modo de acción [13] o de acuerdo con los síntomas observados en humanos tras una intoxicación [14]. En este último caso se consideran cinco síndromes causados por varios grupos de toxinas marinas: intoxicación paralizante por consumo de molusco (PSP, paralytic shellfish poisoning), intoxicación diarreica por consumo de molusco (DSP, diarrhetic shellfish poisoning), intoxicación amnésica por consumo de molusco (ASP, amnesic shellfish poisoning), intoxicación neurotóxica por consumo de molusco (NSP, neurotoxic shellfish poisoning) e intoxicación ciguatérica por consumo de pescado (CFP, ciaguateric fish poisoning). Con el continuo descubrimiento de nuevas toxinas como azaspirácidos (AZAs), gimnodiminas

(GYMs) y espirólidos (SPXs), se ha visto que un mejor enfoque es clasificar las toxinas en base a sus propiedades químicas o estructura química, en vez de sus síntomas tóxicos [15,16]. En función del grado de solubilidad en compuestos orgánicos, las toxinas se agrupan en hidrofílicas y anfifílicas y lipofílicas [17] y basado en su estructura química, las toxinas se pueden clasificar en: grupo del ácido domoico, (DA), grupo del ácido ocadaico (OA) incluyendo las dinofisistoxinas (DTXs), AZAs, brevetoxinas (BTXs), ciguatoxinas (CTXs), iminas cíclicas (CIs), maitotoxinas (MTXs), palitoxinas (PlTXs), pectenotoxinas (PTXs), grupo de las saxitoxinas (STXs), tetrodotoxinas (TTXs) y yessotoxinas (YTXs). En la presente memoria se utilizarán estas dos últimas clasificaciones, indicando las equivalencias con la nomenclatura sintomatológica (tabla 1).

<table>
<tr><th>Toxinas lipofílicas</th><th>Toxinas hidrofílicas</th><th>Toxinas anfifílicas</th></tr>
<tr><td>OA y DTXs (DSP)</td><td rowspan="3">DA y análogos (ASP)</td><td rowspan="3">MTXs</td></tr>
<tr><td>AZAs</td></tr>
<tr><td>BTXs (NSP)</td></tr>
<tr><td>YTXs</td><td rowspan="2">STXs (PSP)</td><td rowspan="3">PlTXs</td></tr>
<tr><td>PTXs</td></tr>
<tr><td>CTXs (CFP)</td><td rowspan="2">TTXs</td></tr>
<tr><td>CIs: SPX, GYMs, PnTXs, PtTXs</td></tr>
</table>

Tabla 1: Clasificación de los principales grupos de toxinas marinas. Entre paréntesis se indica el síndrome causado por la intoxicación de ese grupo de toxinas.

1.1.2. Toxinas hidrofílicas

▪ Grupo del ácido domoico (DA)

Las toxinas del grupo del DA causan el síndrome de intoxicación ASP. El primer episodio tóxico se describió en el año 1987 cuando 143 personas con dolores gastrointestinales y desórdenes en el sistema nervioso central ingresaron en distintos hospitales en Montreal, Canadá, debido al consumo de mejillones (*Mytilus edulis*) [18]. Los pacientes mostraban los síntomas típicos de la intoxicación ASP que se caracteriza por desórdenes intestinales, incluyendo náuseas, vómitos,

calambres abdominales y diarrea que aparecen 24 horas después del consumo de los mejillones. Posteriormente estos pacientes, mostraron síntomas neurológicos como dolor de cabeza, confusión, desorientación, pérdida de memoria a corto plazo, ataques y estado de coma en 48-72 horas. Finalmente, 4 pacientes murieron como resultado de esta intoxicación [18,19]. La toxina causante de este envenenamiento fue el DA. Este compuesto (figura 1) es un ácido amino tricarboxílico, de naturaleza polar, soluble en agua e insoluble en disolventes orgánicos. También se han identificado varios análogos presentes en las diatomeas y en algunos moluscos: los ácidos isodomoicos A, B, C, D, E y F y el C5' diasteroisómero.

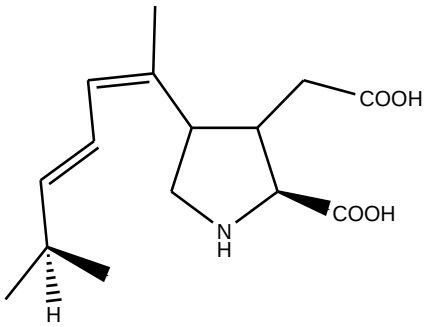


Figura 1: Representación esquemática del DA.

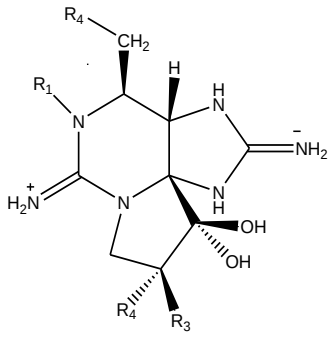
El mecanismo de acción del DA se caracteriza por la activación de los receptores de glutamato en el sistema nervioso central. El DA presenta una gran afinidad por el receptor alfa-amino-3-hidroxi-5-metil-4-isoxazolpropiónico y las subunidades de los receptores de kainato que están presentes en el sistema nervioso central de los mamíferos [20]. La interacción de DA y estos receptores de glutamato provoca la despolarización de la célula y como consecuencia, disfunción y muerte celular [21]. A diferencia de otras toxinas producidas por dinoflagelados, el DA es producido por diatomeas como *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia pungens*, *Pseudo-nizschia psiudodelicaissima*, *Pseudo-nitzschia australis*, *Pseudo- nitzschia seriata* y *Pseudo-nizschia delicatula* [22]. Su distribución no sólo está limitada al norte del continente americano ya que se han encontrado en otros lugares como Nueva-Escocia, Oregón, Washington, Columbia Británica, Japón, Nueva Zelanda y Europa [23,24].

▪ **Grupo de las Saxitoxinas (STXs)**

El grupo de las STXs es el causante del síndrome PSP, una de las intoxicaciones más peligrosas y extendidas del mundo. Son neurotoxinas producidas por varias especies de dinoflagelados entre las que destacan las del género *Alexandrium* (*A. tamarense* y *A. catella*), *Gymnodinium catenatum* y *Pyrodinium bahamense*. El primer dinoflagelado que se relacionó con PSP fue *Alexandrium catenella*, el cual en 1927, cerca de San Francisco, provocó la muerte a 6 personas y síntomas en 102. Se han identificado varias especies de los géneros *Alexandrium*, *Gymnodinium catenatum*, y *Pyrodinium bahamense* var *compressum* como productores de toxinas PSP [25].

Las STXs se unen de modo específico y reversible al sitio 1 de los canales de sodio dependientes de votaje [26], bloqueando el flujo pasivo de iones sodio hacia el interior de la membrana celular e impidiendo la conducción de los potenciales de acción. De este modo, las STXs afectan a la excitabilidad de los nervios y los músculos [27].Cuando los niveles de intoxicación son medios o moderados se produce un estado de malestar generalizado, parálisis facial, astenia, ataxia, disnea, hipotensión, taquicardias, vómitos, disfagia, cefaleas y desordenes gastrointestinales. En caso de intoxicaciones severas puede ocurrir parada respiratoria y/o shock cardiovascular [28]. Los síntomas se manifiestan de 30 minutos a 24 horas después de la ingesta [29].

Desde el punto de vista estructural, las toxinas PSP se caracterizan por ser tetrahidropurinas (figura 2). Poseen un único anillo tricíclico denominado 3, 4, 6-trialquiltetrahidropurina, los derivados resultan de la sustitución en 4 radicales por diversos grupos hidroxilo y sulfato. Según la carga neta que presentan a pH 7, se clasifica en tres grupos [28]: STXs con una carga neta +2, Gonyautoxinas (GTXs) con una carga neta de +1 y N-sulfocarbamoil-11-hidroxisulfatotoxinas (Cs) sin carga. El compuesto más representativo de este grupo es la STX, es el más estudiado y el primero en ser aislado a partir de *Saxidomus gigantescus*, de la cual recibe su nombre. Otro criterio para su clasificación se basa en el tipo de sustituyente en el radical R4, de este modo se distinguen toxinas carbamato, sulfocarbamoil, decarbamoil y deoxicarbamoil.



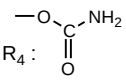
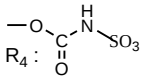
R_1	R_2	R_3	R_4 : 	R_4 : 	R_4 :OH	R_4: H
H	H	H	STX	GTX5 (B1)	dcSTX	doSTX
OH	H	H	neoSTX	GTX6 (B2)	dcneoSTX	-
OH	OSO_3^-	H	GTX1	C3	dcGTX1	-
H	OSO_3^-	H	GTX2	C1	dcGTX2	doGTX2
H	H	OSO_3	GTX3	C2	dcGTX3	doGTX3
OH	H	OSO_3	GTX4	C4	dcGTX4	-

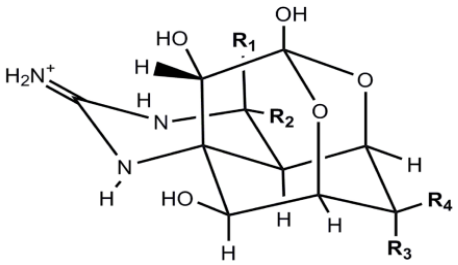
Figura 2: Estructura de STX y sus análogos.

Las toxinas PSP son las toxinas de mayor distribución mundial, afectan al Norte y Sur del continente americano y también a toda Europa, Sudáfrica, India, Marruecos y la costa este de Asia [30]. Se eliminan rápidamente de los tejidos del mejillón mediante la depuración con aguas limpias, sin embargo en algunas especies de almejas, permanecen hasta dos años [28]. No se inactivan con el calor aunque la estabilidad puede variar dependiendo de cada toxina y del pH [14,30].

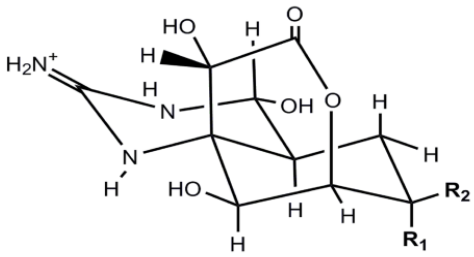
- **Tetrodotoxinas (TTXs)**

Las TTXs son unas de las toxinas más potentes aisladas hasta el momento. Al igual que las STXs, las TTXs actúan bloqueando los canales de sodio responsables de la excitabilidad muscular y nerviosa [31]. Su molécula tiene 6 residuos hidroxilo en las posiciones C-4, C-6, C-8, C-9, C-10 y C-11 y un grupo guanidina que se carga positivamente en el rango de pH biológico. A pesar de que los hidroxilos del

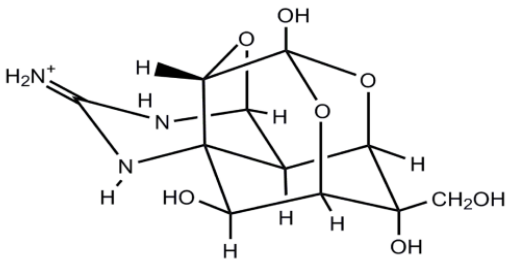
C-9 y C-10 son los más importantes, los que están en C-4, C-6 y C-11 también presentan contribuciones importantes en la unión del canal como donantes de enlaces de hidrógeno [32]. En la figura 3 se representa la estructura química de la TTX y sus principales análogos.



	R1	R2	R3	R4	MH^+ (*m/z*)
TTX	H	OH	OH	CH_2OH	320
4-*epi*TTX	OH	H	OH	CH_2OH	320
11-deoxyTTX	H	OH	OH	CH_3	304
11-norTTX-6(S)-ol	H	OH	OH	H	290
11-norTTX-6(R)-ol	H	OH	H	OH	290



	R1	R2	MH^+ (*m/z*)
5-deoxyTTX	OH	CH_2OH	304
5,6,11-trideoxyTTX	H	CH_3	272



4,9-anhydroTTX MH^+ *m/z* 302

Figura 3: Estructura química de la TTX y sus análogos.

La TTX generalmente coexiste con una mezcla de sus análogos en peces tetraodontiformes y peces globo [33] y es causa de intoxicaciones y muertes en humanos después de la ingestión [31,34]. Los síntomas incluyen entumecimiento de la lengua, labios, parestesia de la cara y las extremidades, seguido de una sensación de mareo y aturdimiento, dolor de cabeza y dolor de estómago, náuseas, diarrea y vómitos. En casos severos inconsciencia, parálisis respiratoria y convulsiones [35].

Muchos casos de intoxicaciones se han registrado en el sureste de Asia y más concretamente en Japón. También se han dado intoxicaciones en Taiwán, Tailandia, Malasia, Singapur, Hong Kong, Australia, Madagascar, China y Bangladesh [35,36]. Varios estudios han revelado la presencia de TTX en animales terrestres y marinos como tritones, peces globo, pulpos, estrellas de mar *y* caracolas [36,37].

1.1.3. Toxinas anfifílicas

- **Maitotoxinas (MTXs)**

Las MTXs son una familia de poliéteres de estructura plana y de gran peso molecular (figura 4) [38]. Inicialmente se consideraron CTXs debido a que son producidas por la misma especie de dinoflagelados y a que se acumulan en tejidos de los peces. Sin embargo, las diferencias estructurales y farmacológicas hacen que actualmente se clasifiquen como un grupo aparte. Las MTXs son producidas por especies de *Gambierdiscus toxicus* y se transmiten por la cadena alimentaria hasta las vísceras de peces herbívoros, pero a diferencia de las CTXs, no pasan al tejido muscular, por lo que su consumo, y por tanto los episodios tóxicos, son menos frecuentes [39].

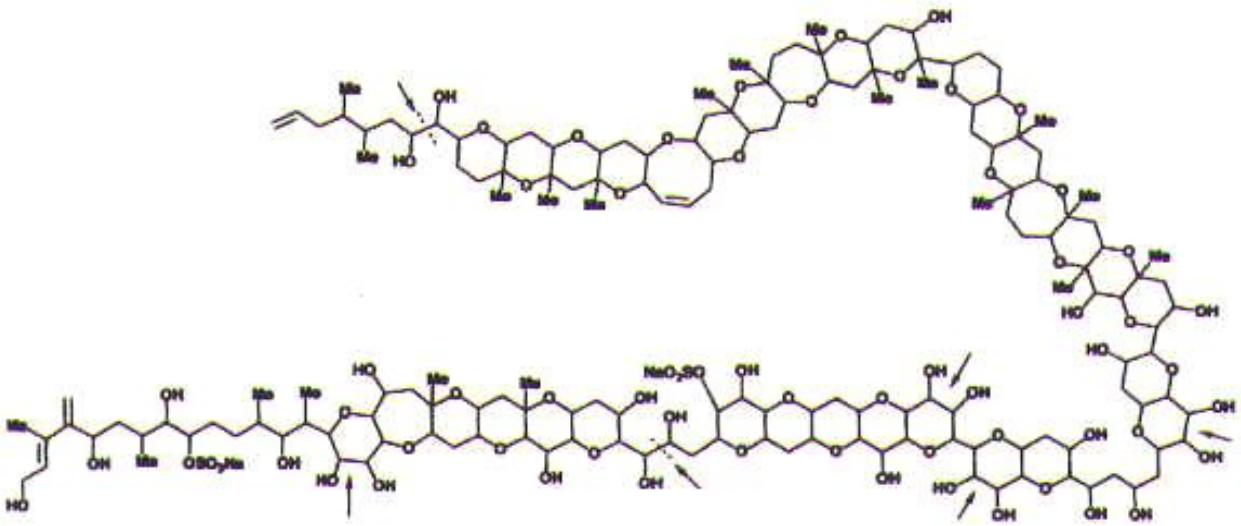


Figura 4: Estructura de la MTX.

Las MTXs actúan como potentes activadores de los canales de calcio de la membrana plasmática en muchos modelos celulares [40] y constituyen uno de los compuestos tóxicos de naturaleza no proteica más potentes [41]. No se han descrito síntomas sobre una intoxicación por MTX separados de los síntomas causados por una intoxicación por CTXs. Siempre se han asociado junto con las CTXs provocando procesos de CFP ciguatera. En estudios toxicológicos llevados a cabo con ratones se observó que la MTX afecta a numerosos órganos, ocasionando en el sistema cardio-vascular necrosis celular y disminución de la luz vascular, múltiples erosiones y úlceras en estómago e intestino y necrosis masiva de los linfocitos del timo [42]. A pesar de ser una de las toxinas naturales más potentes conocidas, el hecho de que su toxicidad por vía oral sea unas 100 veces inferior que por vía intraperitoneal (i.p.) y de que sólo se acumule en las vísceras de los peces, favorece que los episodios tóxicos en los que se ha visto implicada no revistan gran gravedad [39].

- **Palitoxinas (PlTXs)**

Las toxinas del grupo de las PlTXs son producidas por anémonas coralinas del género *Palythoa* (*P. tuberculosa*, *P. Toxica*, *P. Vestitas*, *P. Craibdea*, *P. Mamillosa*) y por varias especies de dinoflagelados del género *Ostreopsis*. Su nombre se debe a la especie de coral Hawaiano de la que fue aislada: *Palythoa toxica* [43].

Estructuralmente la PlTX es una gran molécula no peptídica y polar formada por una cadena de más de 100 carbonos con 64 centros estereogénicos (figura 5). Por lo tanto, se trata de una molécula muy compleja con un gran número de isómeros. Entre sus análogos se pueden citar ostreocina-D, homoPlTX, bis-homoPlTX y deoxiPlTX [44].

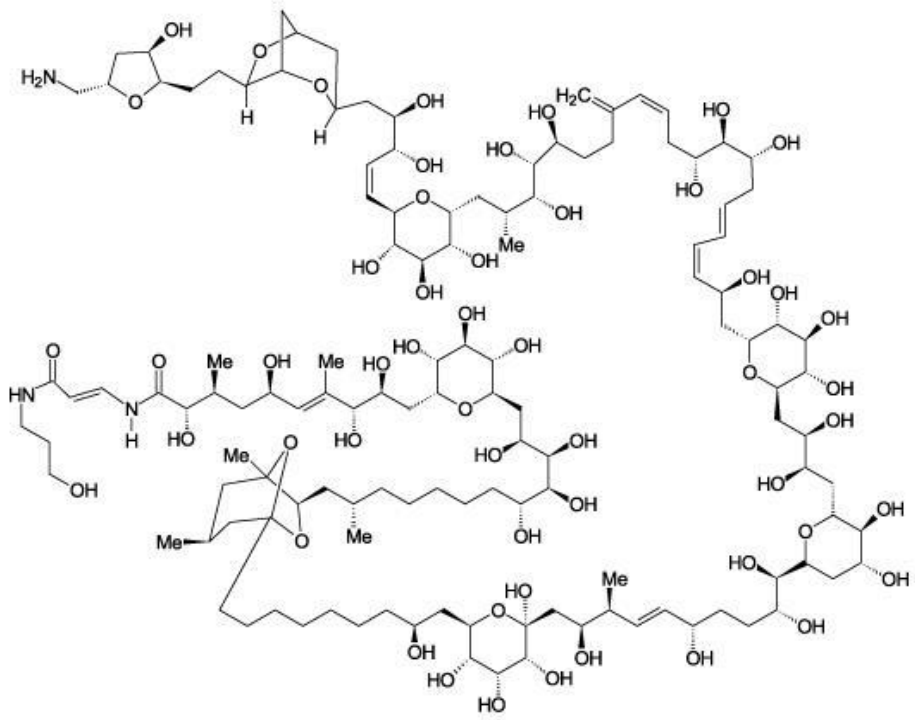


Figura 5: Estructura de la PlTX.

La PlTX es la toxina no peptídica más potente identificada hasta el momento. Inicialmente la PlTX y sus análogos eran producidos por especies localizadas en zonas tropicales y subtropicales pero en la actualidad estas especies se han encontrado en el mar Adriático [45] y la costa mediterránea [46]. Se acumulan en numerosos organismos como corales, esponjas, mejillones y crustáceos. Entre los años 1987 y 1999 se han registrado en Japón al menos 20 incidentes de intoxicaciones humanas debido al consumo de pescado, con 75 pacientes afectados de los cuales 6 murieron. Los alimentos contaminados con PlTX parecen presentar un sabor amargo y metálico, lo que evita que se ingieran grandes cantidades, aún así, debido a su elevada toxicidad son frecuentes los casos mortales.

La principal diana biológica de la PlTX parece ser la bomba ATPasa Na^+ / K^+ de la membrana plasmática, que participa en el mantenimiento de los gradientes iónicos de trans-membrana de las células animales y que es esencial en numerosas funciones celulares [47,48]. La unión de la PlTX inhibe el funcionamiento de la ATPasa y produce la formación de un poro, permeable a cationes monovalentes,

destruyendo así el gradiente iónico en la membrana citoplasmática. Estas alteraciones de la membrana celular desencadenan una serie de señales intracelulares que pueden dar lugar a contracción del músculo liso y esquelético y varios efectos biológicos adversos [49]. Casi inmediatamente tras el consumo del alimento, se manifiestan síntomas de toxicidad como diarrea, vómitos y náuseas, con letargias leves o agudas. Varias horas después de la ingestión los pacientes muestran sensación de quemazón en la zona perioral y en las extremidades, espasmos, temblores, dolor muscular severo, pudiendo llegar al infarto coronario [50].

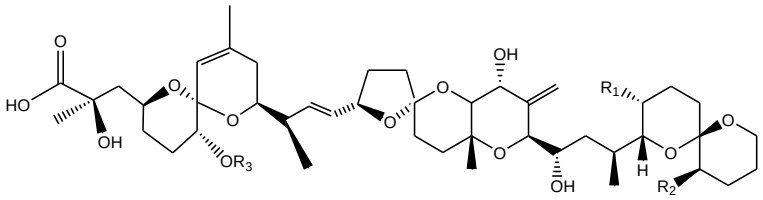
1.1.4. Toxinas lipofílicas

- **Grupo de ácido okadaico (OA)**

El grupo del OA también se conoce como toxinas diarreicas o toxinas DSP. Estas toxinasse registraron por primera vez en Japón en 1976 [51]. El dinoflagelado idenficado como productor fue *Dinofisis fortii*, en poco tiempo le siguió *D. acuminata*, *D. acuta*, *D. norvegica*, *D. mitra, D. ratundata* y *Prorocentrum lima*. Entre 1976 y 1982 se documentaron en Japón más de 1.300 casos de DSP, en 1981 en España más de 5.000 casos y en 1983 sobre 3.300 casos en Francia [52]. Este tipo de intoxicación provoca transtornos gastrointestinales como diarrea, nauseas, vómitos y dolores abdominales. A menudo se pueden confundir con infecciones gástricas bacterianas [22]. Los síntomas aparecen entre 30 minutos y pocas horas después del consumo de los moluscos y se manifiestan durante un máximo de 3 días [52]. Además, algunas de las toxinas involucradas pueden ser promotoras de tumores en el estómago y por tanto producir enfermedades crónicas en los consumidores [22].

Este grupo está formado por el OA y sus derivados las DTXs, todos ellos son compuestos polietéreos liposolubles. En la figura 6 se representa el OA y tres DTXs, la DTX-1, DTX-2 Y DTX-3. El OA es un derivado poliéter de un ácido graso de 38 carbonos con un grupo carboxilo y 4 grupos hidroxilo. Entre las DTXs se pueden diferenciar varios análogos, los derivados libres (DTX-1 y DTX-2) y los esterificados (DTX-3, DTX-4, DTX-5, DTX-6 y otros ésteres diol). Algunas de estas toxinas predominan más en unos lugares que en otros, como es el caso de DTX-2

en las costas inglesas, DTX-1 en Japón o el OA en España [53] y todas ellas son estables al calor, a la congelación y a la salinización [53].



OA	R1 = CH3	R2 = H	R3=H
DTX-1	R1 = CH3	R2 = CH3	R3=H
DTX-2	R1 = H	R2 = CH3	R3=H
DTX-3	R1, R2 =H o CH3		R3 = acilo

Figura 6: Estructura del OA y análogos.

Se ha demostrado que el OA, DTX-1 y DTX-2 son inhibidores específicos de lasfosfatasas de proteínas de residuos de serina y de treonina PP1 y PP2A [53]. Estas fosfatasas están implicadas en la regulación de múltiples procesos celulares como el metabolismo, el transporte y la secreción en la membrana, y la división celular. Este mecanismo de acción es probablemente el responsable de la inflamación del tracto intestinal, diarreas y efecto promotor de tumores [54].

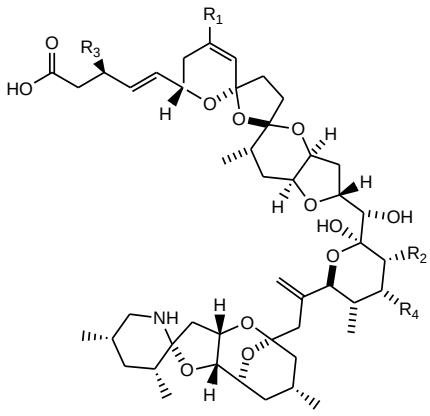
Algunos organismos cuya dieta básica son dinoflagelados tóxicos, podrían ser capaces de destoxificar OA y DTXs, mediante transformaciones químicas. Éste es el caso de la transformación de DTX-1 en DTX-3 por la vieira *Patinopecten yessoensis* [55]. Estas formas aciladas (DTX-3), además de ser menos tóxicas que las moléculas no esterificadas, nunca se han encontrado en microalgas marinas, por lo que se cree que son originadas en los mariscos bivalvos por acilación [56].

- **Azaspirácidos (AZAs)**

Los AZAs son moléculas lipofílicas que presentan tres uniones entre anillos de tipo espiro, una de las cuales agrupa un grupo azaspiro con un anillo 2,9, dioxobiciclononano y en el otro extremo de la molécula presentan un grupo carboxílico (figura 7). Las primeras intoxicaciones en humanos atribuidas a este

grupo sucedieron en Holanda en el año 1995 debido al consumo de mejillones (*Mytilus edulis*) recogidas en a la costa oeste de Irlanda (Kilary Harbour) [57]. Los principales síntomas (nauseas, vómitos, diarrea y dolores de estómago) recordaban a los cuadros clínicos por DSP. Sin embargo, los bajos niveles de estas toxinas detectados en los análisis químicos [58], los síntomas de neurotoxicidad observados en el bioensayo en ratón (MBA) y la presencia de una estructura que no posee ninguna otra toxina marina, el grupo azaspiro [59], indicaron que se trataba de otro compuesto. A estas observaciones se le sumó el hecho de que la toxicidad en los mejillones persistía durante un periodo de tiempo que no es frecuente en un episodio tóxico por DSP, al menos 8 meses. Por todos estos motivos, estas toxinas se incluyeron en un grupo nuevo [60].

El segundo suceso tóxico originado por AZAs ocurrió en octubre de 1997 en el noroeste de la costa de Irlanda en la pequeña isla de Arranmore [60]. Al menos 12 personas enfermaron por el consumo de mejillones y nuevamente su periodo de toxicidad fue de 8 meses. En este caso, junto a azaspirácido-1 (AZA1) se identificaron otros análogos el AZA2 y AZA3 y en menor cantidad el AZA4 y AZA5 que son dos derivados hidroxilados menos frecuentes en la naturaleza [58]. Estudios posteriores revelaron la existencia de más de 20 análogospertenecientes a este grupo, con derivados dihidroxi-, carboxi-, carboxi-hidroxi-, y dehidro- AZAs, así como ésteres de metilo [61,62].



	R_1 (C_8)	R_2 (C_{22})	R_3 (C_3)	R_4 (C_{23})
AZA1	H	CH_3	H	H
AZA2	CH_3	CH_3	H	H
AZA3	H	H	H	H
AZA4	H	H	OH	H
AZA5	H	H	H	OH
AZA6	H	H	CH_3	H
AZA7	CH_3	OH	H	H
AZA8	CH_3	H	H	OH
AZA9	H	OH	CH_3	H
AZA10	H	H	CH_3	OH
AZA11	CH_3	OH	CH_3	H

Figura 7: Estructura de AZAs y sus análogos.

En cuanto al origen de los AZAs, en los primeros estudios se detectaron AZA1, AZA2 y AZA3 en células de la especie *Protoperidium crassipes*. Posteriormente, se demostró que el primer productor de AZAs es el dinoflagelado *Azadinium spinosum*[63]. Se apunta a que los derivados hidroxilados AZA4 y AZA5 podrían ser originados por la bioconversión que el molusco realiza de los compuestos primarios, lo que explicaría la poca abundancia de estos análogos en la naturaleza [58]. En los últimos años además de detectarse en mejillones de la especie *M. edulis* se han encontrado cantidades elevadas de AZAs en otros bivalvos como ostras (*Crassostrea gigas*, *Ostrea edulis*), almejas (*Tapes phillipinariun*), berberechos (*Cardium edule*), y navajas (*Ensis sikiqua*) [64]. Los AZAs persisten durante mucho tiempo en los tejidos, dificultando el proceso de depuración. Aunque se trata de un tipo de molécula estable *in vivo*, varios estudios químicos demostraron que son inestables *in vitro y* que se degradan a elevadas temperaturas [62]. Además, la descomposición de la toxina ocurre en algunos disolventes, particularmente en cloroformo y en soluciones alcalinas ligeras [59].

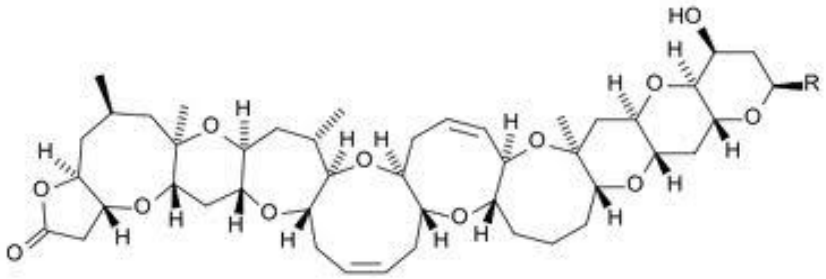
Además de las intoxicaciones anteriormente citadas causadas por AZAs, posteriormente se detectaron otros sucesos tóxicos en Irlanda, Reino Unido, Noruega, Holanda, Francia, España e Italia [64]. Los síntomas observados en humanos son estrictamente gastrointestinales. Estudios de toxicidad aguda llevados a cabo en ratones revelaron que la administración oral del análogo AZA1 ocasiona múltiples alteraciones morfopatológicas diferentes de las inducidas por otras toxinas, como daños en el intestino delgado, necrosis celular en el timo y bazo y degeneración grasa del hígado [65]. Además en el desarrollo del MBA se observan signos de neurotoxicidad con dificultad respiratoria, espasmos, parálisis y convulsiones antes de la muerte. Estudios de toxicidad crónica con AZA1 muestran que la administración de dosis subletales por vía oral ocasionan lesiones epitelieles en estómago e intestino delgado, tumores en el pulmón e hiperplasia del epitelio del estómago que sugieren propiedades tumorgénicas [66]. En cuanto al mecanismo de acción de estas toxinas, hay varios trabajos que apuntan a que la diana de esta toxina está relacionada con el metabolismo de los lípidos [67], kinasas apoptóticas [68,69] o que se une a proteínas [70,71]. De momento no se conoce el mecanismo de acción de estas toxinas.

- **Brevetoxinas (BTXs)**

Las BTXs causan la intoxicación neurotóxica por consumo de molusco NSP. Son neurotoxinas cíclicas de naturaleza polietérea y se asocian con episodios de grandes mortandadesde peces, aves y mamíferos marinos [14]. El dinoflagelado que las produce es *Gymnodinium breve,* originario de las costas de Florida y del golfo de México. Además, se han descrito nuevas especies de dinoflagelados capaces de sintetizar toxinas similares a las neurotóxicas (*Chatonella marina*, *Chatonella antiquea*, *Fibroxapsa japónica* y *Heterosigma akashiwo* y nuevas especies del género *Gymnodinium*) ampliándose el área de distribución a Brasil, España, Japón y Nueva Zelanda [72].

Estructuralmente, las BTXs son poliéteres liposolubles formados por un esqueleto de 10 anillos éter con una lactona en uno de sus extremos (figura 8). En función de los sistemas de anillos de la zona central del esqueleto se clasifican en BTX-A (o de tipo 1) y en BTX-B (o de tipo 2). Dentro del tipo 1 se incluyen los análogos más abundantes de la naturaleza, las BTXs 2 y 3, y las formas 5 y 6, 8 y 9. Dentro del tipo 2 se encuentran los análogos 1, 7 y 10.Todas estas toxinas son estables al calor, a la congelación y a la salinización [14,53].

(A)



(B)

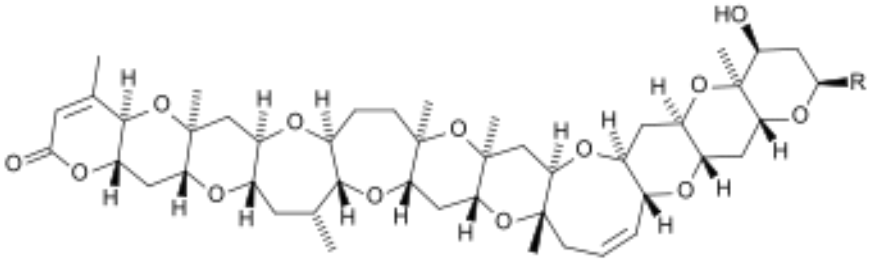


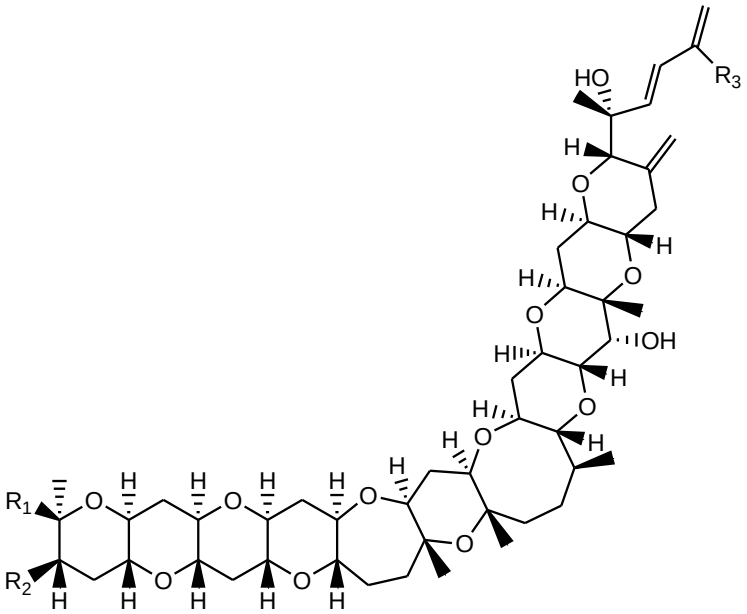
Figura 8: (A) BTX-A y (B) BTX-B.

El síndrome NSP se debe principalmente a la despolarización de la célula por acción de las BTXs, a través de la activación de los canales de sodio dependientes de voltaje. De esta forma, se alteran las propiedades de la membrana de las células excitables favoreciendo el flujo de iones Na^+ hacia el interior de la célula, inhibiendo así las transmisiones neuronales en el músculo esquelético [73,74]. Los principales síntomas de NSP en humanos se caracterizan por náuseas, sensación de hormigueo, pérdida del control motor y dolor muscular severo. Son síntomas de carácter neurológico pero de menor gravedad que los producidos por las toxinas del grupo de las STXs [30]. La recuperación tiene lugar en 2 o 3 días sin que hasta el momento se hayan registrado casos mortales.

- **Yessotoxinas (YTXs)**

La YTX se aisló por primera vez en Mutsu Bay, Japón, en 1986[75] de las glándulas digestivas de la vieira *Patinopecten yessoensis,* de la que ha adquirido su nombre. Posteriormente se encontró en el mejillón *Mytilus edulis* en Noruega [76], en el mejillón *Mytilus galloprovincialis* del mar Adriático en Italia [77], enlos mejillones *Perna canaliculus* (*Greenshell mussel*) de Nueva Zelanda,*Mytilus chilensis* de Chile [78], así como en *Mytilus galloprovincialis*de las rías gallegas [79]. La YTX y sus análogos son producidos por especies de dinoflagelados *Protoceratium reticulatum* y *Lingulodinium polyedrum*.

La YTX es un poliéter disulfatado que posee un esqueleto de 47 carbonos en forma de escalera, formada por 11 anillos éter contiguos, una cadena lateral terminal insaturada de 9 carbonos y 2 ésteres de sulfato (figura 9) [75]. Generalmente se incluye dentro de toxinas lipofílicas, aunque puede ser considerada toxina anfifílica por algunos autores [17]. Además de la YTX se han descrito numerosos análogos incluyendo sus estructuras y configuraciones, [80-82]. Hasta el momento se han identificado y caracterizado por resonancia magnética nuclear (RMN) y cromatografía liquida con detección por espectrometría de masas (LC-MS/MS) 36 derivados naturales, aunque se han descrito más 90 [81].



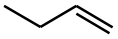
YTX $R_1 = NaO_3SO(CH_2)_2$ $R_2 = NaO_3SO$ $R_3 =$ 

Figura 9: Estructura de YTX.

En cuanto al mecanismo de acción, se ha demostrado que la YTX modula la homeostasis del calcio en linfocitos humanos [40,83] y disminuye los niveles intracelulares de la adenosina fosfato cíclico a través de la activación de las fosfodiesterasas celulares en estas mismas células [84,85]. Además, la YTX produce apoptosis en células de neuroblastoma y en células HeLa por activación de caspasas [86], e induce la disrupción del sistema E-caterina-catenina en células epiteliales [87]. A pesar de todos estos procesos, las YTXs no provocan efectos tóxicos en humanos.

- **Pectenotoxinas (PTXs)**

Las PTXs son un grupo de toxinas del tipo poliéter lactona (figura 10). Se han detectado en microalgas y moluscos bivalvos en Australia, Japón y Nueva Zelanda y en varios países europeos como Italia, Irlanda, Portugal y España [88]. Hasta la fecha se han aislado y caracterizado 15 compuestos con distintos radicales y disposición espacial [89]. También existen derivados ácidos de la PTX-2 como el

ácido seco PTX-2, su epímero 7-epi-PTX-2 y sus ésteres que se caracterizan porque el anillo de lactona está hidrolizado [90,91].

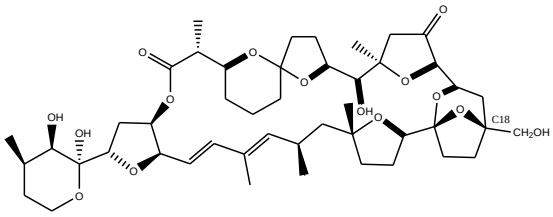


Figura 10: Representación esquemática de PTX-1

Al igual que el OA y las DTXs, las PTXs son producidos por los dinoflagelados del género *Dinophisis,* por esta razón con frecuencia coexisten con los derivados del OA en moluscos contaminados. El primer productor identificado fue *Dinophisis forte* (*D. forte*), pero posteriormente las PTXs se encontraron en *D. acuminata*, *D. rotundata*, *D. acuta*, *D. caudata*, *D. norvegica* [92-94] .Debido a la asociación con el OA y las DTXs, las PTXs han sido tradicionalmente incluidas en el grupo de las toxinas DSP, sin embargo, varios estudios en animales indican que las PTXs son mucho menos tóxicas por vía oral y no inducen diarrea. Por esta razón y porque presentan una estructura química diferente a la del OA, las PTXs han sido clasificadas como un grupo independiente. Las PTXs no inhiben a las fosfatasas de proteínas PP2A y PP1 [95] y no se conoce su diana intracelular. Sin embargo, se ha demostrado un efecto sobre el citoesqueleto de actina [96-98]

Algunos análogos como la PTX-6 se forman a partir de la PTX-2 en tejidos de moluscos. Aunque han sido descubiertas en glándulas digestivas de vieiras, *Patinopecten yessoensis*, contaminadas con DSP [99], también se acumulan en otros moluscos bivalvos filtradores como ostras y mejillones.

- **Ciguatoxinas (CTXs)**

Las CTXs son toxinas marinas producidas principalmente por dinoflagelados del género *Gambierdiscus*, como *G.pacificus, G. toxicus, G. polynesiensis. A*unque también las pueden producir otros microorganismos como *Prorocentrum concavum*, *Prorocentrum mexicanum*, *Procrocentrum rhathytum*, *Gymnodinium sangieneum* y

Gonyaulax poliedra [100]. Estas toxinas se producen fundamentalmente en las regiones del Pacífico, Caribe e Índico y se acumulan en la cadena alimentaria, pasan desde los pequeños peces herbívoros que se alimentan en los arrecifes de coral a los órganos de los grandes peces carnívoros que se alimentan de ellos [101]. Las CTXs causan el síndrome CFP, que se caracteriza por una amplia variedad de síntomas gastrointestinales, neurológicos y cardiovasculares. Como *Gambierduscus*está principalmente distribuido alrededor de los trópicos entre la latitud 32° N y 32°S, consecuentemente el síndrome CFP está mayormente ligado a regiones de los océanos Pacífico, Índico occidental y del mar Caribe. Los síntomas gastrointestinales incluyen vómitos, diarrea, náuseas y dolor abdominal y ocurren típicamente al comienzo de la enfermedad. Los síntomas neurológicos se producen siempre e incluyen hormigueo en labios, manos y pies, percepción térmica alterada y una gran picazón de la piel. CFP es el tipo de intoxicación alimentaria debida a toxinas marinas más común en todo el mundo, con un número estimado de 10.000 a 50.000 intoxicaciones anuales [101].

Químicamente las CTXs son compuestos polietéreos, solubles en lípidos y formadas por 13 ó 14 anillos unidos mediante enlaces éter que generan una estructura rígida (figura 11). Inicialmente se les dio el nombre de gambiertoxinas a los compuestos producidos directamente por el dinoflagelado, reservándose el término CTXs para los derivados de la biotransformación de las sustancias originales en su paso por la cadena trófica. Actualmente se ha ampliado el término de CTXs incluyendo también a las gambiertoxinas [102]. Se han caracterizado varios análogos de CTXs que reciben distinto nombre dependiendo de la localización: una parte procede del Caribe (C-CTXs), otra del Pacífico (P-CTXs) y otra del Índico (I-CTXs). En la tabla 2 se muestran los análogos más comunes de cada localización. Las CTXs son relativamente estables al calor, su toxicidad permanece después de la cocción, la congelación o la exposición a condiciones de acidez suave y básica [103].

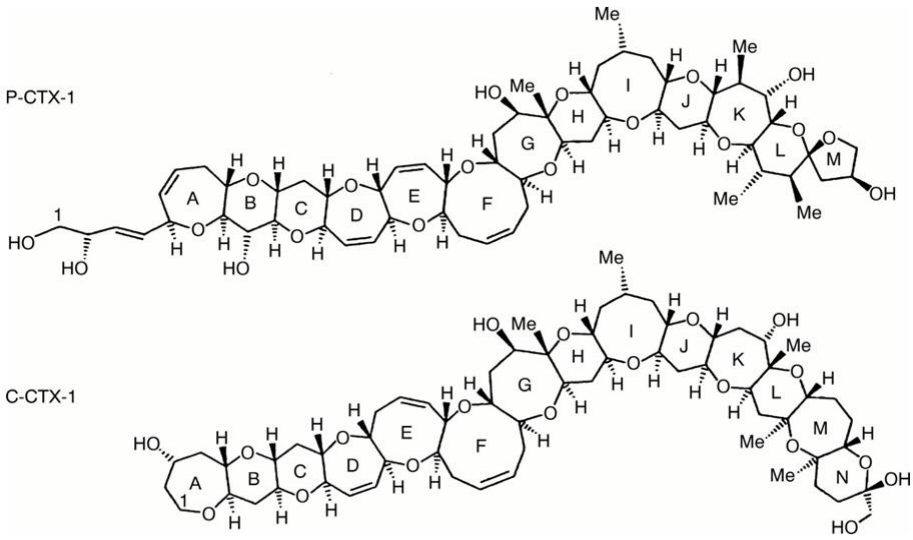


Figura 11: Estructura de P-CTX-1 y C-CTX-1.

Ciguatoxina	Peso molecular	Referencia
P-CTX-1	1110,6	[104]
P-CTX-2	1094,7	[104]
P-CTX-3	1126,7	[104]
P-CTX-3C	1022,5	[105]
P-CTX-4A	1060,5	[106]
P-CTX-4B	1060,5	[106]
2,3-dihydroxy P-CTX-3C	1056,5	[107]
51-hydroxy P-CTX-3C	1038,5	[107]
C-CTX-1	1040,6	[108]
C-CTX-2	1040,6	[108]
I-CTX-1	1040,6	[109]
I-CTX-2	1040,6	[109]
I-CTX-3	1156,6	[109]
I-CTX-4	1156,6	[109]

Tabla 2: Principales análogos de las CTXs.

La información toxicológica para este grupo de toxinas comprenden principalmente estudios de toxicidad aguda llevados a cabo por inyección i.p. [101]. En general son más tóxicas las P-CTXs que las C-CTXs. La P-CTX-1, P-CTX-2 y P-CTX-3 tienen una dosis letal 50 (LD_{50}) de 0,25, 2,3 y 0,9 µg/kg, respectivamente [110]. Sin embargo, la LD_{50} de C-CTX-1, es 3,6 µg/kg [111]. El mecanismo de acción de las CTXs se asocia con la apertura de los canales de sodio dependientes de voltaje en las células excitables. Se ha comprobado que su actividad sobre el canal de sodio es inhibida competitivamente por otras ficotoxinas marinas, como las BTXs, por lo que es posible que al igual que ellas, las CTXs ejerzan su acción al unirse específicamente al sitio 5 del canal de sodio con alta afinidad [39].

Se conoce poco sobre la distribución de las especies de *Gambierdiscus.* Por lo general, el área de distribución de este dinoflagelado se sitúa en las zonas del Pacífico y del Caribe. Por ejemplo, *G. belizeanus*, *G. carolinianus*, *G. ruetzleri* and *G. riotypes* son exclusivos del océano Atlántico y *G. australes*, *G. pacificus*, *G. polynesiensis*, *G. toxicus* y *G. yasumotoi* han sido encontradas solo en el océano Pacífico. Sin embargo en un estudio reciente sobre la distribución de este dinoflageladose observó que algunas otras especies como *G. carpenteri* y *G. caribaeus* tienen una distribución global [112]. En general, las concentraciones altas de *Gambierdiscus* se asocian con elevadas temperaturas del agua pero también con tipos de desastres naturales como huracanes, vientos fuertes y entradas de nutrientes [112]. En los últimos años, la presencia de *Gambierdiscus* se ha registrado en la zona este del Mediterráneo. El primer registro de la presencia de *Gambierdiscus* en esta zona se dio en la isla de Creta en 2003 [113]. Posteriormente, en enero de 2004 una familia de pescadores tuvo síntomas de CFP después de la ingesta de parte de un pescado, de la especie *Seriola rivoliana*, de 26 Kg capturado en la costa de las islas Canarias. Una muestra de 150 g de este pescado se conservó en el congelador y se analizó por un ensayo *in vitro* específico para toxinas que actúan sobre los canales de sodio y por LC-MS/MS. Los resultados de los análisis fueron positivos y el contenido de CTX en la muestra resultó ser de 1,0 µg/kg [114]. Años más tarde, también se confirmó la presencia de organismos que producen CTXs en el Mediterráneo, en la costa de Israel [115] y de nuevo en las islas Canarias [116]. Estos hallazgos sugieren una expansión de estas toxinas a nuevas áreas y por consiguiente, su aparición y en peces y moluscos europeos.

▪ **Iminas cíclicas (CIs)**

Este grupo está formado por SPXs, GYMs, pinnatoxinas, pteriatoxinas, prorocentrolides y spiro-prorocentrimines. Son compuestos macrocíclicos formados por grupos imina y grupos éter con uniones tipo espiro. Debido a su similitud en estructura química y en toxicidad en ratones, todas estas toxinas se consideran en el mismo grupo. Los datos toxicológicos para las CIs son limitados y comprenden solo estudios de su toxicidad aguda tras administración i.p. o administración oral con sonda. Las CIs bloquean los receptores de acetilcolina en el sistema nervioso periférico y central, incluyendo uniones neuromusculares [117].

● **Espirólidos (SPXs)**

Los SPXs son el grupo más grande de las CIs. Actualmente se conocen 12 análogos, de los cuales el 13-desmetil SPX C (13-desMeC) es el más común en los moluscos (figura 12). La primera vez que se detectaron fue en mejillones y almejas de Nueva Escocia (Canadá) durante la primavera y el verano de 1991 [118,119]. En esta época también se registraron síntomas en humanos como taquicardia y dolores gastrointestinales después del consumo de los moluscos contaminados. El organismo identificado como el productor de estas toxinas fue *Alexandrium ostenfeldii* (*A. ostenfeldii*). Hasta entonces, este organismo considerado una especie de agua fría [120], solo se había descrito como un productor de neurotoxinas associadas a PSP [121,122] ya que varias especies del género *Alexandrium* producían toxinas PSP. Sin embargo, después de este episodio, *A. ostenfeldii* se identificó como el principal productor de SPXs [123].

En la última década, *A. ostenfeldii* ha aparecido en aguas templadas de todo el mundo con la peculiaridad de que las toxinas que producen pueden ser diferentes dependiendo de la región de la que proceda el dinoflagelado. Por ejemplo, en la costa de Canadá, golfo de Maine, USA y en el norte del mar Adriático en Italia, *A. ostenfeldii* produce SPXs [120]. Sin embargo, en la costa de Nueva Zelanda, este organismo solo produce toxinas PSP [124] y finalmente, en Dinamarca y países escandinavos, se encontraron ambos grupos de toxinas en el mismo periodo de tiempo [121]. Los SPXs se han detectado en un gran número de especies y localidades en todo el mundo. Además de Canadá, USA (golfo de Maine) [125], Dinamarca [122] e Italia [126], se han detectado también en España [127], Francia [128], Irlanda [129], Escocia [130], Noruega [131], y Chile [132].

Además del anillo imina heptacíclico, los SPXs contienen grupos éter cíclicos unidos por grupos espiro (figura 12). Basado en su estructura química, estos compuestos se dividen en 4 grupos. En el primero se engloban los SPXs que, además del anillo imina heptacíclico, tienen un sistema de 3 anillos éter de 5 y 6 lados unidos por grupos espiro (unión 6-5-5). Esta clase incluye SPX A, B, C, D, 13-desMeC, 13,19-didesmetil espirólido C (13,19-didesMeC), 13-desmetil espirólido D (13-desMeD), 27-hidroxi-13,19-didesmetil SPX C (27-OH-13,19-didesMeC). El segundo grupo de SPXs tiene el mismo sistema de anillos éter pero el anillo imina heptacíclico está abierto dando lugar a unaamina. Esta clase comprende SPX E y F. El tercer grupo incluye SPX G y su análogo 20-metil-espirólidoG (20-MeG). Ambos tienen el anillo imina heptacíclico y un inusual sistema de anillos trispiroketal-5-5-6, el cual no ha sido encontrado en otros grupos de toxinas marinas [122]. Posteriormente, se ha descrito el cuarto grupo comprendido por los SPXs H e I [133]. Los 2 contienen un sistema de anillos dispiroketal-5-6 en lugar del sistema de anillos trispiroketal característico de los SPXs previamente descritos. A pesar de que el grupo imina se considera un grupo funcional que se hidroliza con facilidad [134], se ha demostrado que en los SPXs del grupo C, D y G (SPX C, D, G, 13-desMeC, 13,19-didesMeC, 13-desMeD, 27-OH-13,19-didesMeC y 20-MeG) el grupo imina es completamente estable a la hidrólisis ácida y a la hidrólisis enzimática en el molusco [134,135]. Estos SPXs contienen un grupo metil adicional que no poseen los SPXs A y B.

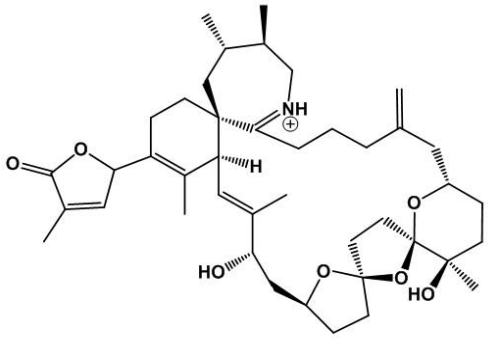


Figura 12: Estructura del 13-desmetil-SPX-C.

Estudios iniciales sobre el mecanismo de acción de los SPXs, revelaron que el 13-desMeC causa una sobreexpresión de receptores muscarínicos (mAChR) y nicotínicos (nAChR) de acetilcolina [136]. Posteriormente, se demostró que los

SPXs se unen a los nAChR neuronales con afinidades subnanomorales ejerciendo un antagonismo potente [3] y también que se une a los mAChRs produciendo una reducción de la función, una disminución de la unión de antagonista específicay alteraciones del receptor en células de neuroblastoma humano [137]. Hasta hace poco tiempo, la existencia del grupo imina cíclico se consideraba un elemento indispensable para que los SPXs presentaran toxicidad [117]. Sin embargo, se ha descrito que el SPX H, que contiene el grupo imina cíclico intacto, no muestra toxicidad en el MBA, lo que sugiere que la presencia de la imina cíclica no es solo el único requisito para la toxicidad [133]. Hay pocos estudios de toxicidad por vía i.p en ratones. Se sabe que la LD_{50} por vía i.p. es de 40 µg/kg para una mezcla de SPXs (fundamentalmente de 13-desMeC) [119]. Mientras que muestras puras de 13-desMeC, SPX C, y 20-MeG tienen una LD_{50} de 6.9, 8.0 y 8.0 µg/kg, respectivamente [117]. Los SPX B y D presentan la misma potencia tóxica con una dosis letal 100% (LD_{100}) de 250 µg/kg [138]. El SPX A, presenta una toxicidad mucho menor con una LD_{50} de 250 µg/kg [139]. Los SPXs E y F, que no poseen grupo imina cíclico, son mucho menos tóxicos y con dosis inferiores a 1 mg/kg no se observa ningún efecto adverso. Por vía oral, los SPXs son mucho menos tóxicos que cuando se inyectan por vía i.p. Hay varios estudios en los que se administran distintos análogos de estos compuestos a ratones por medio de una sonda y en presencia de comida y los resultados son muy distintos según el modo de administración. Los SPXs muestran una mayor toxicidad cuando se administran con sonda que en presencia de comida. En animales intubados, la LD_{50} del SPX C, 13-desMeC y 20-MeG se sitúa entre 53 y 176 µg/kg [117]. En el caso de la administración en presencia de comida, los valores de LD_{50} para el SPX C y 13-desMeC se encuentran entre 500 y 780 µg/kg y para el 20-MeG entre 500 y 625 µg/kg [117].

- **Gimnodiminas (GYMs)**

La primera referencia histórica de la GYM, es de 1994 en Nueva Zelanda, donde controles rutinarios para la detección de toxinas liposolubles presentaron una toxicidad neurológica inusual [140]. Durante el mismo periodo, se observó un florecimento de *Gymnodinium mikimotoi,* por loque el compuesto bioactivo que se aisló de ostras contaminadas, se denominó GYM [141]. Posteriormente, ese dinoflagelado se identificó como *Gymnodinium selliforme* y finalmente renombrado como *Karenia selliformis* [142]. En la actualidad este organismo es el único

productor conocido de estas toxinas. A parte de Nueva Zelanda, también se ha registrado presencia de GYM en moluscos de Túnez [143] y de Australia [144].

La toxicidad en humanos de este compuesto es desconocida, pero estudios en ratones muestran que la GYM es mucho más tóxica por vía i.p. (LD_{50} en el rango 80-96 µg/kg [145,146] que por vía oral, donde cantidades de7500 µg/kg suministrados en presencia de comida no provocan ningún signo de toxicidad [145]. Actualmente se conocen dos análogos más, la gymnodimina B (GYM-B) y la gymnodimina C (GYM-C) (figura 13) [147,148], ambas aisladas de cultivos de *Karenia selliformis*. Y a la GYM inicialmente descubierta se le denomina actualmente gymnodimina A (GYM-A) (figura 13) [149]. Estudios recientes en ratones muestran que la GYM-B presenta una LD_{50} por vía i.p. de 800 µg/kg [146]. No existen datos de toxicidad para GYM-C.

La diana principal de estas ficotoxinas son los receptores colinérgicos nicotínicos, tanto musculares como neuronales, a los que se unen con afinidades en el rango nanomolar [145,146].

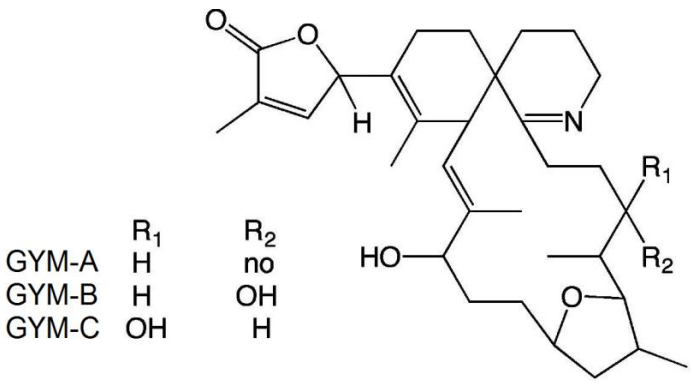


Figura 13: Estructura de las GYMs.

● **Pinnatoxinas (PnTXs)**

Las PnTXs son las CIs que presentan la estructura química más cercana a los SPXs. Este subgrupo está formado por siete análogos (A, B, C, D, E, F y G). Durante muchos años, estas toxinas sólo se aislaron de moluscos japoneses, almeja *Pinna muricata*[150], pero en los últimos años han aparecido en Australia y Nueva Zelanda [151], en Noruega [152] y en Canadá [153]. Por vía i.p. estas

toxinas presentan una dosis letal 99% (LD_{99}) en ratones de entre 22 y 400 µg/kg [12]. Estudios iniciales con extractos de la almeja *Pinna attenuata*, sugerían que estos compuestos eran activadores de canales de calcio [154], sin embargo se ha demostrado que actúan inhibiendo los nAChR de manera irreversible [155].

- **Pteriatoxinas (PtTXs)**

El grupo de las PtTXs está formado por tres análogos: A, B y C [156]. Su nombre proviene de la ostra *Pteria penguin*, de donde se aislaron en 2001 [41]. A pesar de que esta especie de ostras está ampliamente distribuida, las PtTXs solo han aparecido en moluscos japoneses [117,151]. Existe la hipótesis que *Pteria penguin* y *Pinna muricata* acumulan las mismas toxinas precursoras pero las metabolizan de forma distinta [157]. La toxicidad i.p. en ratón, LD_{99}, varía entre los 8 y los 100 µg/kg peso corporal [41]. No existen datos de toxicidad oral ni de su mecanismo de acción.

- **Prorocentrolidos**

Los prorocentrolidos, A y B, son las CIs más grandes, con una masa molecular alrededor de 1000 Da. Los prorocentrolidos A se aislaron de *Prorocentrum lima* [158] y los prorocentrolidos B de *Prorocentrum maculosum* (anteriormente *Prorocentrum concavum*) [159]. A menudo se encuentran junto al OA y sus derivados. No existen datos fiables sobre la toxicidad de estos compuestos ni sobre su mecanismo de acción.

- **Espiro-prorocentrimina**

Esta toxina única de su subgrupo, se aisló de una cepa bentónica de *Prorocentrum sp*. en Taiwan [160]. El único dato de toxicidad existente es su LD_{99} por vía i.p. en ratones, que equivale a 2500 µg/kg [160]. No existen datos sobre su mecanismo de acción.

1.2. Métodos de detección de toxinas marinas

El riesgo asociado con el consumo de moluscos ha obligado a las autoridades de todo el mundo a instalar sistemas de monitoreo que impiden que los moluscos con toxinas pasen a los mercados e intoxiquen a los consumidores. El monitoreo de toxinas marinas se ha convertido en una cuestión importante, no sólo desde un punto de vista sanitario, sino también desde el punto de vista económico ya que la presencia de floraciones de toxinas en aguas controladas conlleva a cerrar las áreas de producción, con las consecuentes pérdidas económicas. En este sentido, es muy importante emplear el menor tiempo posible en el análisis e interpretación de los resultados utilizando métodos de detección rápidos y eficaces. En las últimas décadas se han desarrollado una serie de métodos analíticos que comprenden ensayos *in vivo* como el MBA, ensayos *in vitro* (celulares, de receptores, de inhibición enzimática e immunoensayos) y métodos químicos que incluyen análisis por cromatografía líquida de alta eficacia (HPLC) con detección por ultravioleta (UV), por fluorescencia (FLD) y por espectrometría de masas (MS) [161]. En el mundo, la legislación de las toxinas marinas difiere de unos países a otros. En Japón, Canadá y Sudamérica, el método utilizado para el control oficial de la mayoría de las toxinas es el MBA, mientras que en Nueva Zelanda se utilizan métodos químicos para los programas de monitoreo [162,163]. La legislación europea reconoce distintos métodos de detección para varios grupos de toxinas citados anteriormente pero otros muchos no están regulados. En la siguiente tabla (tabla 3) se recogen los grupos de toxinas que están regulados en la Unión Europea (UE), los métodos oficiales para su detección y los límites reglamentarios según la legislación actual. En general, además de los métodos de análisis biológicos deben aceptarse métodos de detección alternativos, como son los métodos químicos y los ensayos *in vitro*, si se demuestra que son tan eficaces como el método biológico y que su aplicación proporciona un nivel equivalente de protección de la salud pública. El método de referencia para la detección oficial de las toxinas lipofílicas durante años fue exclusivamente el método del MBA. Pero desde julio del 2011 se reconoce el método químico (tecnología LC-MS/MS) como método de referencia para la detección de las toxinas lipofílicas [164]. Después de 3 años de convivencia de ambos métodos, en el 2014, la técnica de la LC-MS/MS será el único método oficial.

	Grupos de toxinas	Métodos oficiales	Toxinas reguladas	Límites
HIDROFÍLICAS	DA	HPLC y ELISA	DA y análogos (DA, iso-DA A, D, E, F and epi-DA)	20 mg DA/kg
	STX	HPLC y MBA	STX y análogos (dcSTX, GTX1,4, GTX2,3, GTX5, C1,2, C3,4, NEO)	800 µg eq STX/kg
LIPOFÍLICAS	YTXs	MBA y LC-MS/MS	YTX, 45-OH-DTX, homoYTX, 45-homoYTX	1 mg eq YTX/kg
	AZAs		AZA-1, AZA-2, AZA-3	160 µg eq AZA/kg
	OA		OA, DTX-1, DTX-2, DTX-3	160 µg eq OA/kg
	PTXs		PTX-1, PTX-2	

Tabla 3: Toxinas reguladas en la UE, métodos oficiales para su detección y límites para cada uno de los grupos de toxinas.

- **Grupo del DA**

El grupo del DA se pueden detectarporHPLCo por el ensayo de inmunoabsorción ligado a enzimas (ELISA) [165,166]. Si los resultados por ambos métodos son contradictorios, el método de referencia debe ser el HPLC [165]. El hecho de que la estructura del DA presente un grupo dieno conjugado hace que presente un pico de absorción a λmax = 242 nm lo que facilita la determinación por HPLC seguido por la detección UV (HPLC-UV). El primer método de HPLC-UV se desarrolló en 1989 después de una extracción con metanol al 50% acuoso [167]. En 1991, se propuso un método de HPLC-UV basado en una extracción con ácido clorhídrico (HCl) 0,1 M [168]. Este análisis por HPLC-UV se mejoró posteriormente combinando la extracción con metanol al 50% acuoso con un paso de limpieza adicional [169]. También es posible medir el DA con un método de HPLC con detección por FLD (HPLC-FLD). Este método se basa en una derivatización pre-columna con, por ejemplo, 9 fluorenilmetilcloroformato para formar el fluorenilmetoxicarbonilo derivado o 4-fluoro-7-nitro-2,1,3-benzoxadiazol, que se detecta por HPLC-FLD [170,171]. Este método se desarrolló en un primer lugar para monitorizar el DA en el agua del mar y fitoplancton, así como para la detección de DA en extractos de moluscos [172]. También se han desarrollado varios métodos de HPLC, utilizando la detección MS [173-175]. El otro método oficial, ELISA, es un método simple y altamente sensible que permite la cuantificación simultánea y rápida de un gran número de muestras [176]. El ensayo se basa en el reconocimiento específico de la toxina por anticuerpos que se unen a ella. El complejo toxina-anticuerpo se detecta y se mide con la ayuda de un anticuerpo

marcado con una enzima. Se añade un reactivo no coloreado, y el enzima produce una reacción de color donde la intensidad de color es directamente proporcional a la concentración de la toxina en la muestra. El color se mide por absorbancia. Este método es efectivo para la determinación de la concentración de DA y para un cribado [176]. Después de su comercialización en 1998 [177] este método fue mejorado [178]. Actualmente hay un kit comercial con el que se puede detectar el DA con un límite de detección de 10 mg / kg de molusco. Siendo el límite oficial 20 mg / kg de carne [179]. El grupo de expertos en la cadena alimentaria de la Autoridad Europea de Seguridad Alimentaria (EFSA) basándose en los datos disponibles sobre toxicidad aguda en humanos han establecido una dosis de referencia aguda para el DA de 30 mg/kg peso corporal [180].

- **Grupo de las Saxitoxinas (STXs)**

El grupo de las STXs se puede detectar por MBA como método de referenciao por HPLC (método de Lawrence) [165,181]. La primera vez que se utilizó el MBA para la determinación de toxinas PSP fue en 1937 para analizar extractos ácidos de mejillón [182]. Posteriormente, el protocolo se estandarizó y se validó a través de una serie de estudios intercomparativos. Este método de referencia es reconocido internacionalmente para cuantificar toxinas PSP y se usa en los programas de monitoreo de todo el mundo [183]. El MBA para toxinas PSP comprende la extracción del tejido (cuerpo entero u órganos seleccionados) en medio acuoso ácido. La detección se realiza inoculando 1 mL del sobrenadante del extracto en tres ratones por vía i.p. y se monitoriza la sintomatología y el tiempo transcurrido hasta la muerte del animal. La toxicidad de la muestra se expresa en unidades de ratón (1 unidad de ratón se define como la cantidad mínima necesaria para provocar la muerte de un ratón de entre 18 y 22 g de peso en 15 minutos) y se calcula según las curvas de respuesta a las dosis obtenidas con estándares de STX. Entre los métodos químicos el único que ha sido validado internacionalmente por la AOAC para la detección de las PSP es el método de Lawrence [168]. Es un método de HPLC con detección por FLD y con derivatización precolumna (usando peróxido de hidrógeno y periodato) [184]. Este método es aplicable a la determinación de STX, NEO, GTX-2 y 3 (juntas), GTX-1 y 4 (juntas), dc-STX, GTX5 (B-1), C-1 y C-2 (juntas), y C-3 y C-4 (juntas) en moluscos bivalvos (mejillones, ostras, almejas y vieras). Otro método químico no oficial empleado para la determinación de biotoxinas PSP es el método de Oshima 1984 [185]. Este método

está basado en una oxidación alcalina postcolumna para producir derivados fluorescentes. Actualmente, en la UE el límite máximo permitido de estas toxinas en moluscos destinados al consumo humano es de 800 µg STX equivalentes/ kg de carne de molusco (cuerpo entero o partes comestibles) [186]. La EFSA ha establecido una dosis de referencia aguda para la STX y análogos de 0,5 mg eq. STX/ Kg y ha apuntado que no es posible hacer una estimación fiable del riesgo que supone el consumo de moluscos en el mercado porque hay una alta proporción de muestras en las que no se detecta las STXs debido a que los métodos analíticos tienen unos límites de detección altos [187].

- **Toxinas lipofílicas**

El método de referencia para la detección oficial de las toxinas lipofílicas durante años fue exclusivamente el método del MBA. En Europa, desde julio del 2011 se reconoce la tecnología basada en la LC-MS/MS como método de referencia para la detección de las siguientes toxinas lipofílicas: toxinas del grupo del OA (OA, DTX-1, DTX-2 y DTX-3), toxinas del grupo de las PTXs (PTX-1 y PTX-2), toxinas del grupo de la YTX (YTX, 45 OH YTX, homo YTX, y 45 OH homo YTX) y toxinas del grupo de los AZAs (AZA-1, AZA-2 y AZA-3) [164]. Después de 3 años de convivencia de ambos métodos, a partir de 2014, la técnica de la LC-MS/MS será el único método oficial. Este método está orientado a detectar distintas toxinas marinas en un corto periodo de tiempo [162,188] y así poder usarse en programas de monitoreo. En general, este método es específico y permite la detección simultánea de varias toxinas con un límite de detección (LOD) y un límite de cuantificación (LOQ) bajos. Actualmente hay un protocolo aprobado por el Laboratorio de Referencia Europeo para biotoxinas marinas [189] donde se describe las condiciones del análisis. El método utiliza una cromatografía en fase reversa en una columna de sílica C8 o C18 y un gradiente de elución o isocrático con fases móviles a base de acetonitrilo (ACN) y agua que contienen modificadores volátiles como ácido acético, ácido fórmico, formiato amónico o acetato de amonio. La detección por MS se lleva a cabo en modo de monitoreo de reacciones múltiples (MRM) usando dos transiciones por toxina. La transición que presenta la intensidad más alta se usa para la cuantificación de la toxina, mientras que la transición con baja intensidad se usa para confirmar el compuesto. Con la excepción del grupo del OA y de la YTX que se ionizan en modo de ionización negativo, el resto de las toxinas lipofílicas se

ioniza en modo positivo. Si el instrumento es capaz de trabajar en modo simultáneo positivo y negativo, los métodos de MS incluyen las transiciones para las toxinas que se ionizan en ambos modos. Si el equipo no es capaz, los compuestos se detectan en dos cromatogramas, uno operando en modo positivo y el otro en modo negativo. Los parámetros de MS deben estar optimizados con estándares de toxinas para conseguir el máximo nivel de sensibilidad en los análisis. Hasta la fecha, se han descrito sobre 200 toxinas lipofílicas [162]. Sin embargo, este método de LC-MS/MS está enfocado al análisis de las toxinas reguladas en la UE. En la tabla 4 se recogen las principales características del método químico para detectar las toxinas lipofílicas por LC-MS/MS.

Condiciones de LC	Columna	BDS-Hypersil C8, 50 mm x 2 mm, 3µm		
	Flujo	0.2 mL/min		
	Volumen de inyección	5 µL		
	Tª de la columna	25 ºC		
	Gradiente	Tiempo (min)	Fase móvil A (%)	Fase móvil B (%)
		0	70	30
		8	10	90
		11	10	90
		11.5	70	30
		17	70	30
Transiciones MS/MS	**Compuesto**	ESI	Q1 > Q3	Q1 > Q3
	OA / DTX-2	neg	803.5 > 255.0	803.5 > 113.0
	DTX-1	neg	817.5 > 255.0	817.5 > 113.0
	YTX	neg	1141.5 > 1061.7	1141.5 > 855.5
	45 OH-YTX	neg	1157.5 > 1077.7	1157.5 > 871.5
	HomoYTX	neg	1155.5 > 1075.5	1155.5 > 869.5
	45 OH-HomoYTX	neg	1171.5 > 1091.5	1171.5 > 869.5
	PTX-1	pos	892.5 > 821.5	892.5 > 213.2
	PTX-2	pos	876.5 > 823.4	876.5 > 213.2
	AZA-1	pos	842.5 > 824.5	842.5 > 806.5
	AZA-2	pos	856.5 > 838.5	856.5 > 820.5
	AZA-3	pos	828.5 > 810.5	828.5 > 792.5

Tabla 4: Principales características del método LC-MS/MS para la detección de las toxinas lipofílicas. La fase móvil está compuesta de agua (A) y ACN/ agua (95:5) (B), ambas conteniendo 50 mM de ácido fórmico y 2 mM de formiato amónico. Las transiciones MS/MS para monitorizar las toxinas están también indicadas en ella.

Otros métodos no oficiales para detectar las toxinas lipofílicas son los métodos funcionales basados en el mecanismo de acción de cada una de ellas. Por ejemplo, para las toxinas del grupo del OA, hay ensayos que utilizan la fosfoprotein-fosfatasa [190-192] que pueden detectar las toxinas a niveles por debajo del límite legislado. Para la YTX hay ensayos basados en interacciones de la este compuesto con las fosfodiesterasas [85,193,194].

Los SPXs y las CTXs no están reguladas por lo que no existen métodos oficiales para su detección. Los SPXs se incluyencon frecuencia en el método oficial de multi-toxinas de LC-MS/MS porque se detectan en las mismas condiciones de cromatografía [195]. Además para los SPXs, se ha utilizado su capacidad de unirse a los nAChR para desarrollar métodos de detección [196]. Para las CTXs, el método químico que se emplea con más frecuencia también es la tecnología de la LC-MS/MS. Los sistemas de cromatografía líquida comprenden columnas C18 como Phenomenex Luna-5 µm [197] o Zorbax 300SB-3.5 µm [109,198] y C18 como Phenomenex Hyperclone [199] o Phenomenex Luna [73]. Las fases móviles están compuestas generalmente de ACN y agua [109,114,199,200]. Además de los métodos químicos, las CTXs se pueden detectar por MBA, descrito por primera vez en 1960 [201] y por ensayos *in vitro*. En este grupo se engloban los ensayos de citotoxicidad [27], los inmunoensayos y los ensayos de unión a receptores [74] que están basados en la inhibición de la unión de una brevetoxina tritiada, [^{3}H]-brevetoxina-3, a los canales de sodio de células de cerebro de rata en presencia de grupos de CTX [74]. Aunque actualmente no existen límites legislados para las toxinas del grupo de las CIs ni de las CTXs, para los SPXs se ha propuesto fijar la cantidad de 400 µg/ kg de carne [202]. Y en lo que respecta a las CTXs, el reglamento de la UE establece que se llevarán a cabo controles para asegurar que los productos pesqueros que contengan CTX no se comercialicen [203,204].

2. Objetivo

La presencia de toxinas lipofílicas es cada vez más frecuente en las costas europeas. Los sistemas oficiales de monitorización y control garantizan la ausencia de estos compuestos en los productos de la pesca destinados al consumo. Sin embargo, la aparición de toxinas nuevas y la presencia de toxinas de otras latitudes en Europa hace necesaria una constante renovación de la información disponible para una actualización de la legislación. En este contexto, los objetivos de la presente tesis doctoral son:

-La purificación de SPXs en alta cantidad a partir del dinoflagelado *Alexandrium ostenfeldii,* para desarrollar un método de detección basado en su mecanismo de acción y estudiar su farmacocinética y toxicidad.

-Estudiar la presencia de CTXs en productos de la pesca recogidos en las costas europeas.

-Evaluar los parámetros críticos del nuevo método oficial de detección de toxinas lipofílicas de la UE basado en la tecnología LC-MS/MS.

3. Publicaciones

3.1. Presentación

En esta sección se presentan los resultados obtenidos en la tesis doctoral que han sido publicados en revistas científicas, en total 7 artículos. En cada artículo se describe la metodología científica utilizada, así como las conclusiones y la discusión de los resultados obtenidos. Las publicaciones que se muestran están relacionadas con la producción, purificación, detección y toxicidad de los SPXs y con la detección de las CTXs y toxinas DSP.

De este modo, los resultados obtenidos se agrupan en tres secciones que se resumen a continuación:

3.2. SECCIÓN I: SPXs. Producción, purificación, detección y estudios de toxicidad *in vivo* de SPXs.

Los SPXs se han detectado en un gran número de localizaciones en todo el mundo, pero el estudio de su mecanismo de acción y de sus efectos tóxicos se ralentizó debido a la escasa cantidad de patrones de esta toxina. *A. ostenfeldii* es el principal productor de SPXs, aunque a menudo produce distintas toxinas del grupo de las PSP [121,124]. Este dinoflagelado aparece en el agua del mar en bajas concentraciones, generalmente menores a 10^3 céls/L. La máxima densidad de células en episodios tóxicos es menor a 4 x 10^3 céls/L en Nueva Escocia [205], 7,7 x 10^3 céls/L en las costas danesas [206] y, 2,2 x 10^3 céls/L en aguas noruegas [131]. Por lo tanto, el desarrollo de un método de purificación de esta toxina y la producción a gran escala a partir de cultivos de *A. ostenfeldii* es de enorme importancia.

A pesar de que no se han descrito intoxicaciones humanas debido a la ingestión de SPXs, su gran recurrencia en numerosos países y los síntomas observados en el MBA indican que estos compuestos constituyen un grupo de toxinas que deberían estar reguladas. Hay varios que apuntan a que los mAChR y los nAChR son la principal diana de los SPXs [136,137]. Pero la falta de datos toxicológicos impide que se fijen límites legislados para estas toxinas y en general para el grupo de las CIs. Y por lo tanto, no se establece un método oficial de monitorización para su detección.

En esta sección se aborda el estudio de las condiciones ambientales en las que se producen estas toxinas, así como el desarrollo de un método de purificación para obtener estos compuestos puros. Se propuso un método de detección funcional para estas toxinas basado en la unión a los nAChR, susceptible de ser utilizado

para medir concentraciones de SPXs en varias muestras, incluyendo mejillones destinados al consumo humano. Por último, se estudió la toxicidad oral e i.p. de los SPXs en ratones y sus efectos toxicológicos. Esta información es muy importante para la regulación en la UE de los SPX.

A esta sección corresponden las siguientes publicaciones:

I.1. Effects of environmental regimens on the toxin profile of *Alexandrium ostenfeldii*.

I.2.New protocol to obtain spirolides from *Alexandrium ostenfeldii* cultures with high recovery and purity.

1.3. First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples.

I.4. Pharmacokinetic and toxicological data of spirolides after oral and intraperitoneal administration.

I.1. Efectos de las condiciones ambientales en el perfil de toxinas de *A. ostenfeldii*

Resumen

Las condiciones ambientales son factores clave en el crecimiento y desarrollo del fitoplancton marino. Este estudio muestra la primera evidencia de que los factores ambientales externos pueden influenciar en el perfil de toxina producida por el dinoflagelado *A. ostenfeldii*. La especie investigada es originaria de la costa del Atlántico Norte y sus células crecieron bajo varios parámetros físicos ambientales. La producción de toxina se midió por LC-MS/MS y los cromatogramas reflejaron la presencia de dos SPXs en todos los cultivos, uno en la región *m/z* 692,5, que corresponde al 13-desMeC y el otro en la región *m/z* 678,5, que corresponde al 13,19-didesMeC. Los parámetros físicos que se estudiaron fueron salinidad, medio de cultivo y fotoperíodo. La cantidad más alta de toxina por célula se obtuvo cuando los dinoflagelados crecieron en medio de cultivo F/2 y Walne, 28 ‰ de salinidad, y 24h de luz. Sin embargo, la mayor proporción de 13,19-didesMeC con respecto a 13-desMeC se obtuvo en medio L1, 33 ‰ de salinidad y 14:10 h luz: oscuridad. Por el contrario, la proporción más alta de 13-desMeC se obtuvo cuando *A. ostenfeldii* se cultivó en medio F/2, 28 ‰ de salinidad y el mismo fotoperíodo. Teniendo en cuenta estos parámetros, en este estudio se mostraron las

condiciones óptimas para cultivar *A. ostenfeldii* y obtener gran cantidad de SPXs por células. Además, esas condiciones ambientales pueden ser consideradas una herramienta para predecir y evitar las floraciones de *A. ostenfeliii.*

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EFFECTS OF ENVIRONMENTAL REGIMENS ON THE TOXIN PROFILE OF *ALEXANDRIUM OSTENFELDII*

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Abstract—Environmental conditions are key factors in the development of marine toxic phytoplankton. Spirolides are marine toxins with a heptacyclic imine ring responsible for the toxicity in mice. *Alexandrium ostenfeldii* (*A. ostenfeldii*) is the main producer of these toxins, although this dinoflagellate often produces toxins belonging to the paralytic shellfish poisoning (PSP) group. The present study shows the first evidence that external environmental factors can influence the toxin profile produced by the dinoflagellate *A. ostenfeldii*. The species investigated is indigenous to the North Atlantic coast, and their cells grew under several environmental parameters. Toxin production was measured by means of liquid chromatography-mass spectrometry (LC-MS) and the chromatograms reflect the presence of two spirolides in all cultures; one in the region *m/z* 692.5, corresponding to 13-desmethyl spirolide C (13-desMeC) and the other in the region *m/z* 678.5, which corresponds to 13,19-didesmethyl spirolide C (13,19-didesMeC). The physical parameters studied were salinity, culture media, and photoperiod. The highest amount of toxin per cell was obtained when dinoflagellates grew in F/2 and Walne medium, 28‰ salinity, and 24 h of light. However, the highest proportion of 13,19-didesMeC with respect to 13-desMeC was achieved in L1 medium, 33‰ salinity, and 14:10 h light:dark. On the contrary, the highest proportion of 13-desMeC in cells was obtained when *A. ostenfeldii* was cultured in F/2 medium, 28‰ salinity, and the same photoperiod. Therefore, from these data the optimum conditions to culture *A. ostenfeldii* and to obtain the highest amount of spirolide per cell are shown. In addition, these environmental conditions can be considered a tool to predict and avoid *A. ostenfeldii* blooms. Environ. Toxicol. Chem. 2010;29:301–310. © 2009 SETAC

Keywords—*Alexandrium ostenfeldii* Spirolides Liquid chromatography-mass spectrometry Paralytic shellfish poisoning High-performance liquid chromatography

INTRODUCTION

Spirolides are phycotoxins belonging to the cyclic imine group that also includes compounds such as pinnatoxins, pteriatoxins, espiro-prorocentrimine, and gymnodimine [1–3]. In these toxins, the cyclic imine moiety is the one responsible for the neurological symptoms, including convulsions, cramps, and rapid death (within minutes), that are observed in mice injected intraperitoneally with toxic lipophilic extracts from shellfish contaminated with spirolides [4,5]. The mode of action is still unknown, but experiments indicate that the muscarinic and nicotinic receptors may be the targets for these toxins [6].

General symptoms, such as tachycardia and stomachache, were reported for the first time following contaminated shellfish consumption from Nova Scotia (Canada) during the spring and summer of 1991, when spirolides were detected in mussels and clams from this region [4,7]. The dinoflagellate identified as the producer of these spirolide toxins was *Alexandrium ostenfeldii* (*A. ostenfeldii*). This organism, considered a cold-water species [8], was described only as a producer of neurotoxins associated with paralytic shellfish poisoning (PSP) [2,3], because several species of genus *Alexandrium* (*A. acatenella*, *A. catenella*, *A. fundyense*, *A. minutum*, *A. tamarense*, *A. tamiyavanichii*) produce only PSP toxins, not spirolide toxins. However, after that episode, *A. ostenfeldii* was identified as the main producer of spirolide toxins [9].

Alexandrium ostenfeldii has been present for the last 10 years in temperate waters throughout the world. In Europe, this species was found in Denmark, Norway, Scotland, Spain, Belgium, and Italy, with the peculiarity that the produced toxins can be different depending on the region from which the dinoflagellate came. For example, on the Canadian coast, the Gulf of Maine, USA, and the Northern Adriatic Sea in Italy, *A. ostenfeldii* produced only spirolide toxins. On the other hand, only PSP toxins were found in this organism on the coast of New Zealand, and finally, both toxin groups were found at the same time in species found near Denmark and Scandinavia.

The macrocyclic structures of spirolides contain spiro-linked tricyclic ether groups in addition to the heptacyclic imine ring. So far, seven compounds associated to this toxin group have been isolated and characterized from dinoflagellates cultures and mollusk extracts. They were designated with letters A, B, C, D, E, and F. Spirolide G has been recently identified [3], as well as some des-methyl derivatives of spirolides C and D, namely, 13-desmethyl spirolide C (13-desMeC) [10], 13,19-didesmethyl spirolide C (13,19-dedesMeC) [3], and 13-desmethyl spirolide D [4]. Finally, a methyl derivative of spirolide G, called 20-methyl spirolide G (20-MeG) [11], and a hydroxilated analogue of 13,19-didesMeC, designated 27-hydroxy-13,19-didesmethyl spirolide C (27-OH-13,19-didesMeC) [12], were also discovered. The structures of commonly known spirolides are presented in Figure 1. Spirolides E and F are

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shellfish metabolites in which the cyclic imines of spirolides A and B have been opened to a keto amine. The main difference in the structure of spirolides from groups A, B, C, and D is the nature of the radical located at C13, C19, and C31. It can be –H or $–CH_3$ depending on the compound. Nevertheless, small changes in the structure of spirolide compounds originate produce significant changes in the toxicity. After oral administration to mice, spirolides C and D are more toxic than spirolides A and B [7], while intraperitoneally 13,19-didesMeC is five times less toxic than spirolide 13-desMeC [3]. The lack of the imine cyclic group in the spirolides E and F gives rise to inactivity in the mice bioassay [4], and the toxicity of spirolide G was not described. This compound contains a 5:6:6-trispiroketal ring system never observed before in other marine toxin groups, which could have repercussions for the molecule toxicity [3].

The most commonly used technique to identify spirolide toxins is liquid chromatography-mass spectrometry (LC-MS) [4]. In the present study, this method was used to detect and quantify known spirolides by comparing them with standards in cultures of *A. ostenfeldii*. It was also used to identify molecules that belong to this group of toxins following specific ions in the mass spectrometry-mass spectrometry spectral.

The present study provides information on the toxin profiles of a strain of *A. ostenfeldii* isolated from the North Atlantic. This strain produced spirolides and PSP toxins, but their quantity and proportions vary depending on the culture conditions. These data were used to determine the optimum conditions for maximum spirolide production. The present study shows the proper environmental parameters to culture the dinoflagellate and produce high quantities of spirolides per cell, never before observed in *A. ostenfeldii* strains.

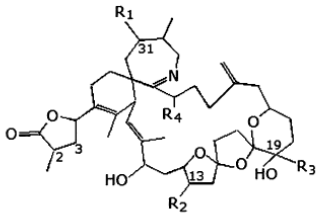
MATERIALS AND METHODS

Reagents and toxin standards

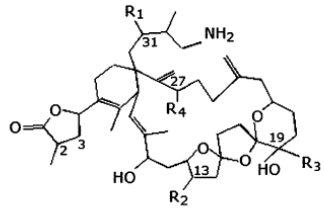
A standard solution of 13-desMeC and the certified PSP standards GTX 1–4 (gonyautoxin 1–4), GTX 2–3 (gonyautoxin 2–3), GTX 5 (gonyautoxin 5), STX (saxitoxin) and neoSTX (neosaxitoxin) were purchased from The Institute for Marine Biosciences, National Research Council. Methanol, dichloromethane, acetonitrile, periodic acid, and 0.03 N acetic acid came from Panreac Quimica S.A. Sodium phosphate, *n*-heptanesulfonic acid, and *o*-phosphoric acid were obtained from Sigma-Aldrich. Ammonium formate was purchased from Merck. All solvents used in this study were high-performance liquid-chromatography or analytical grade, and the water was distilled and passed through a water purification system (Arium 611 Sartorius).

Liquid chromatography-mass spectrometry analysis

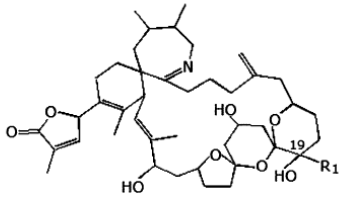
Spirolide toxins were analyzed by means of LC-MS. The HPLC system from Shimadzu consisted of binary LC-10ADVP pumps, a CTO-10AVP column oven, and the injections were performed by a SIL-10ADVP automatic injector. The experiments were performed using Analyst software (Shimadzu). Mass spectral analyses were performed by using an Application Programming Interface triple–quadruple mass spectrometer



R_1	R_2	R_3	R_4	Δ	
H	CH_3	CH_3	H	$\Delta^{2,3}$	Spirolide A
H	CH_3	CH_3	H		Spirolide B
CH_3	CH_3	CH_3	H	$\Delta^{2,3}$	Spirolide C
CH_3	CH_3	CH_3	H		Spirolide D
CH_3	H	CH_3	H	$\Delta^{2,3}$	13-desMeC
CH_3	H	H	H	$\Delta^{2,3}$	13,19-didesMeC
CH_3	H	CH_3	H		13-desMeD
CH_3	H	H	CH_3	$\Delta^{2,3}$	27-OH-13,19-didesMeC



H	CH_3	CH_3	H	$\Delta^{2,3}$	Spirolide E
H	CH_3	CH_3	H		Spirolide F



R_1	
H	Spirolide G
CH_3	20-MeG

Fig. 1. Chemical structures of known spirolides found in shellfish and *Alexandrium ostenfeldii*. Compounds indicated with 2,3 have a double bond between carbons 2 and 3. The molecular weights of spirolide toxins go from 677.5 (spirolide 13,19-didesMeC) to 711.5 (spirolide F).

(QTRAP) equipped with an electrospray ionization (ESI) source. The column used for separations was a 2 × 50 mm BDS-hypersil-C8 analytical column with a particle size of 3 μm and a 10 × 2.1-mm guard cartridge from Thermo. The temperature was set at 25°C. The mobile phase consisted of two components: water (A) and acetonitrile/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 30 to 90% B for 8 min, then 90% B and 10% A were held for 3 min and reduced afterward to 30% B over 0.5 min. Then, they were held again for 2.5 min until the next run. The mobile phase flow rate was 0.2 ml/min and the injection volume was 5 μl.

Collision-induced dissociation in the ion-trap MS was carried out on the protonated molecule $[M + H]^+$, for each toxin. Quantitatative data for two spirolides were based on standard 13-desMeC. For LC-MS calibration, working standard solution was prepared in methanol on the day of analysis and diluted to obtain eight calibration levels within a mass range from 12.5 to 1,000 ng/ml for 13-desMeC. Good linear calibration data were obtained for spirolide standard in solutions ($r^2 = 0.998$).

The parameters depending on the source were optimized and their values were as follows: Curtain gas™ (15), collision-activated dissociation gas (6), IonSpray Voltage (4,000), temperature (450), gas 1 (50), and gas 2 (50).

Analysis of PSP toxins

High-performance liquid chromatography was used to separate the different PSP congeners prior to electrochemical oxidation and fluorescence detection, based on a variation of the method of Oshima [13]. The HPLC is a Shimadzu system equipped with two LC-10ADvp pumps, one LC-6A pump, a SIL-10ADvp automatic injector (10-μL injection loop), a SCL-10Avp system controller, and one RF535 fluorescence detector, set at 340 nm excitation and 410 nm emission wavelengths, from Shimadzu. Data were analyzed with Class-vp software (Shimadzu). The separation was carried out on a reversed phase column (Luna 4.6 × 250 mm, 5 μm). Two types of mobile phases were used to detect different groups of PSP toxins: 2 mM *n*-heptanesulfonic acid, 10 mM *o*-phosphoric acid, and acetonitrile 10% were applied to detect saxitoxin (STX) and their analogues, neosaxitoxin (NEO) and decarbamoylsaxitoxin (dcSTX). Alternatively, 2 mM *n*-heptanesulfonic acid and 10 mM *o*-phosphoric acid were used to detect gonyautoxins (GTX) and their analogues, decarbamoylgonyautoxins (dcGTXs). Both solutions were adjusted to pH 7.2 with ammonium hydroxide.

Samples were evaporated to dryness using an evaporation system (miVac centrifugal concentrator from Genevac). The residues were dissolved in the equivalent volume of 0.03 N acetic acid and passed through 0.45-μm filters (Millipore Ultrafree-MC centrifugal filter units) to be analyzed by HPLC.

Alexandrium ostenfeldii *cultures*

Alexandrium ostenfeldii culture, whose origin was the Kattegat Sea in Denmark, was isolated in 1986. The culture comes from The Provasoli Guillard National Center for Culture of Marine Phytoplankton, strain CCMP1773. The cells were cultured and grown in seawater enriched with four culture media: L1, F/2, Walne, and Algal at two salinity levels (28 and 33‰). The salinity was adjusted at these proportions by the addition of fresh water, removing chlorine by aeration. The cell growth occurred at 18 to 19°C, and some cultures were subjected to a photoperiod with daylight lamps on a 14:10-h light:dark photocycle and others were subjected to 24 h of light. Cells were shaken manually twice a day and growth was divided in steps, increasing the volume progressively. The dinoflagellates were kept under these conditions until the end of the exponential growth phase, and their cells were counted using an Sedgewick-Rafter camera (Lab Safety Supply).

The four culture media, L1, F/2, Walne, and Algal, were chose from the literature, as the media often used to grow dinoflagellates in controlled conditions. These media have different amounts of salts, vitamins, elements, and trace metals. The following is a brief description of each medium.

F/2 medium. For 1 L of filtered seawater we added the following: $NaNO_3$, $NaH_2PO_4{\cdot}H_2O$, $Na_2SiO_3{\cdot}9H_2O$ (molar concentration in final medium 8.83×10^{-4} M, 3.63×10^{-4} M, 1.07×10^{-4} M, respectively), 1-ml F/2 trace metal solution, and 0.5 ml F/2 vitamin solution. The following elements were added to prepare 1 L F/2 trace metal solution: $FeCl_3{\cdot}6H_2O$ (1×10^{-5} M), $Na_2EDTA{\cdot}2H_2O$ (1×10^{-5} M), $CuSO_4{\cdot}5H_2O$ (4×10^{-8} M), $Na_2MO_4{\cdot}2H_2O$ (3×10^{-8} M), $ZnSO_4{\cdot}7H_2O$ (8×10^{-8} M), $CoCl_2{\cdot}6H_2O$ (5×10^{-8} M), $MnCl_2{\cdot}4H_2O$ (9×10^{-7} M). The F/2 vitamin solution (1 L) was prepared by adding the following compounds: cyanocobalamin (1×10^{-10} M), biotin (2×10^{-9} M), and thiamine·HCl (3×10^{-7} M).

Walne medium. To 1 L of sterilized seawater was added 0.1 ml of vitamin solution and 1.0 ml of nutrient solution. The formed solution was made by adding (per 100 ml): cyanocobalamin (10.0 mg), thiamine·HCl (10.0 mg) and biotin (200.0 μg). The later was prepared by adding (per litre): $FeCl_3{\cdot}6H_2O$ (1.3 g), $MnCl_2{\cdot}4H_2O$ (0.36 g), H_3BO_3 (33.6 g), ethylenediaminetetraacetic acid (EDTA) (45.0 g), $NaH_2PO_4{\cdot}2H_2O$ (20 g), $NaNO_3$ (100.0 g), and trace metal solution (1 ml). This trace metal solution (100 ml) was prepared by adding: $ZnCl_2$ (2.1 g), $CoCl_2{\cdot}6H_2O$ (2.0 g), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.9 g), and $CuSO_4{\cdot}5H_2O$ (2.0 g).

Algal medium. First, a concentrated Algal solution was prepared: 53.75 g nutrients were dissolved in 900 ml distiller water (solution A), then a 1.75-g metal and vitamin solution was dissolved in 100 ml distiller water in a separate container (solution B). Both solutions (A and B) were autoclaved and pooled to obtain the concentrated Algal solution (C). Then, 4 ml of C were dissolved in 996 ml sea water. Thus, a final Algal solution had a nitrate concentration of 2 mM. Nutrients composition: Ferric citrate (10.50 g), sodium molybdate (1.16 g), KNO_3 (937.5 g), and NaH_2PO_4 (62.5 g). Metals and vitamins composition: $MnCl_2$ (0.85 g), $ZnCl_2$ (0.63 g), $CoCl_2$ (0.10 g), $CuSO_4$ (0.15 g), EDTA (9.50 g), thiamine (2.10 g), biotin 2% (1.36 g), and cyanocobalamin 1% (1.90 g).

L1 medium. To 1 L of sterilized seawater was added: 0.075 g $NaNO_3$, 0.00565 g, $NaH_2PO_4{\cdot}2H_2O$, 1.0 ml of trace elements stock solution 1, and 1.0 ml of vitamin mix stock solution 2. Stock solution 1 was made by adding (per 1 L): $FeCl_3{\cdot}6H_2O$ (3.15 g), $Na_2EDTA{\cdot}2H_2O$ (4.36 g), $CuSO_4{\cdot}5H_2O$ (1×10^{-8} M), $Na_2MoO_4{\cdot}2H_2O$ (9×10^{-8} M), $ZnSO_4{\cdot}7H_2O$ (8×10^{-8} M), $CoCl_2{\cdot}6H_2O$ (5×10^{-8} M), $MnCl_2{\cdot}4H_2O$ (9×10^{-7} M), H_2SeO_3 (1×10^{-8} M), Na_3VO_4

(1×10^{-8} M), K_2CrO_4 (1×10^{-9} M). Stock solution 2 was made by adding (per 1 L): cyanocobalamin (0.0005 g), thiamine HCl (0.1 g), biotin (0.0005 g).

RESULTS

The type of toxin produced by the dinoflagellate *A. ostenfeldii* varies among different strains, depending on the region of origin. Therefore, the first objective was to determinate the toxin profile produced by a species indigenous to Denmark. The spirolide content of this strain was initially assessed through the analysis of 13 water samples cultured within a few months interval. The dinoflagellates were grown in L1 medium, with daylight lamps on a 14:10-h light:dark photocycle, at 18°C and salinity 33‰. The cells were separated from water by gravity filtration using an 18-cm diameter funnel and a 20-μm pore mesh filter. Then pellets were conserved in methanol with a ratio of three volumes of solvent per cell volume. To confirm the absence of toxins in the filtered water, it was saved to be analyzed later. The extracts were sonicated while cooling in an ice bath and kept at −20°C until analysis. To discover the type and the content of spirolide toxin, the extracts were analyzed by LC-MS.

Before performing the analysis and to achieve the highest sensitivity, several parameters were optimized by standard infusion of 13-desMeC (*m/z* 692.5) on the electrospray ionization source in positive mode at 10 μL/min with a syringe pump. Apart from *m/z* 692.5, the mass spectrum (MS2) of 13-desMeC showed other masses (Fig. 2A). One was *m/z* 674.3, which corresponds to the mass of spirolide standard (*m/z* 692.5) without a water molecule (*m/z* 18). The mass observed at *m/z* 444.4 is a product of the Retro-Diels-Alder reaction of the six-membered ring with a double bond. Multiple water losses observed in the regions *m/z* 674.3 (*m/z* = 656.5, 638.1) and *m/z* 444.4 (*m/z* = 426.4, 408.1) are characteristic of several polyether compounds. Other ions with low intensity were also observed. From these results, the two masses selected as product ions of the 13-desMeC (*m/z* 692.5) were *m/z* 674.3 and 444.4. The next step was to determine the retention time for 13-desMeC by means of multiple reaction monitoring (MRM) mode. Therefore, one injection of standard by the HPLC system in positive mode and at 1,000 ng/mL was made following the transitions: *m/z* 692 > 674 and 692 > 444. As it is reflected in the chromatogram appearing in Figure 2B, the standard 13-desMeC showed an elution time of about 2.03 min. The two peaks observed in this figure are the two transitions followed for 13-desMeC analysis in the cultures, but only *m/z* 692 > 674 was employed to quantify the samples.

Then the methanolic samples described above were analyzed. An aliquot of each sample was filtrated through 0.45-μm filters (Millipore). Mass spectrum for *A. ostenfeldii* extracts showed the presence of two prominent peaks approximately 2 min (Fig. 3A), which could be identified as spirolide toxins, the first at 1.84 min and the second at 2.10 min. These peaks were analyzed separately. The ion eluted at 2.10 min corresponds to a mass of *m/z* 692.5 (Fig. 3B), that is, 13-desMeC toxin, based on the comparison of its molecular weight and retention time with the pure compound. The other peak with the highest intensity at 1.84 min (Fig. 3A) matched with the mass of 13,19-didesMeC (*m/z* 678.5) (Fig. 3B). To confirm that the molecule corresponded to this spirolide, a MS2 product ion was performed (Fig. 4A). Data fragmentation indicated that one ion appeared at the *m/z* 430 to 460 range; it is the *m/z* 430.4 product ion (Fig. 4B). This means that spirolide toxin is following the same fragmentation procedure that gave rise to *m/z* 444.4 at the 13-desMeC. The mass spectrum of 13,19-didesMeC showed a protonated molecule at *m/z* 678.4, in addition to the mass at *m/z* 430.4. The *m/z* 678.4 was followed by a series of ions at *m/z* 660.4, 642.4, and 624.4, which were representative of the sequential loss of water molecules typical of polyether-containing compounds. The cluster of ions in the region of *m/z* 430.4 was also caused by several H_2O losses. Therefore, this molecule is 13,19-didesMeC, and its identification in samples was made by followings the ions: 678.5 > 660.5 and 678.5 > 430.5. In this case, the transition employed to quantify was only the first.

Then a multiple reaction monitoring for each sample was performed by selecting the four objects of the study. As can be observed, in Figure 5, the interferences were eliminated and a clean chromatogram was obtained. In this way, the samples observed in Table 1 (cultures 1–13) were identified and

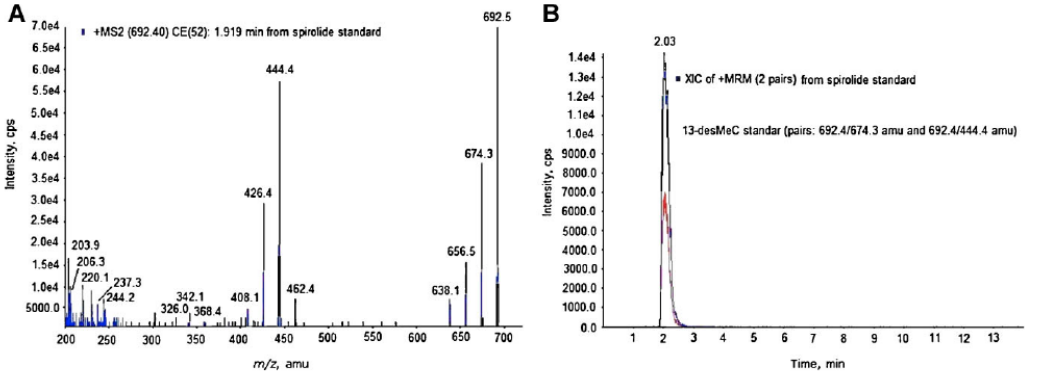


Fig. 2. (**A**) MS2 espectrum and (**B**) multiple reaction monitoring (MRM) chromatogram in positive mode of 13-desMeC standard (1,000 ng/ml) on the triple-quadrupole mass spectrometer (QTRAP) instrument, precursor ion: 692.5, collision energy: 52.00. XIC = extracted ion chromatogram; cps = counts per second; MS = mass spectrometry; amu = mass shift; CE = collision energy. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

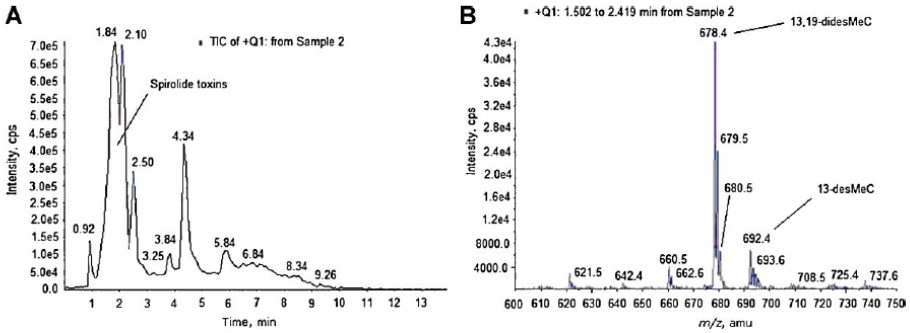


Fig. 3. (**A**) Chromatogram and (**B**) Q1 spectrum of sample 2 from 1.4 to 2 min from the liquid chromatography-mass spectrometry analysis of a methanol extract of *Alexandrium ostenfeldii*. TIC = total ion chromatogram; cps = counts per second; amu = mass shift. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

quantified. The 13-desMeC and 13,19-didesMeC toxins concentration was determined by comparing the analytic standard peak with the peak areas detected in the samples. The cultures contained 4,101.5 ± 368.7 cells/ml and the spirolide content was 2.5 ± 0.6 pg/cell for 13,19-didesMeC and 0.05 ± 0.01 pg/cell for 13-desMeC, whereas the proportion was 97.60 ± 0.28% for 13,19-didesMeC and 2.40 ± 0.28% for 13-desMeC.

The filtrated water was analyzed to check for the presence of toxins. The water was extracted with dichloromethane to yield three extracts, which were concentrated and diluted in methanol to be analyzed by LC-MS. No peak corresponding to spirolide toxins was detected. Therefore, the entire content of toxins was in the cell fraction at the time of filtering.

Some *A. ostenfeldii* strains can produce PSP toxins; this possibility was checked by means of HPLC analysis using Oshima's method. The chromatogram (Fig. 6A) shows the PSP toxin profile in one culture, but all of the cultures had similar PSP toxin profiles. As it is shown, there were traces of GTX and C group toxins in all *A. ostenfeldii* cultures but neither STX nor NeoSTX were found. Figure 6B shows the GTX profile in one culture. The main toxins of this group were GTX1, GTX4, and GTX5, although very low amounts of GTX2 and GTX3 were also found. The analyses for PSP toxins performed by HPLC demonstrated the existence of GTX and C toxins groups in lesser amounts than spirolide toxins.

After identifying the toxins in the cultures, the next step was to study the influence of culture conditions on toxin production. Therefore, more cells growing under other physical parameters were obtained and theirs toxin profiles were compared with the 13 previous. The objective was to know if the external parameters affect to the toxin profile and to propose the optimal culture conditions to obtain high toxin production.

First, the culture media usually employed for this dinoflagellate were studied: F/2, Walne, and Algal media. Nine cultures were grown, and the number of cells and toxin amount produced are represented in Figure 7. Results show that there were not significant differences in the toxin quantity produced by cells cultured with F/2 or Walne growth medium. The amounts were 1.20 ± 0.13 ng/ml for 13-desMeC and 11.56 ± 2.17 ng/ml for 13,19-didesMeC in F/2 medium and 1.13 ± 0.14 ng/ml for 13-desMeC and 11.05 ± 1.81 ng/ml for 13,19-didesMeC in Walne medium. However, the toxin amount obtained with the Algal medium was low (0.49 ± 0.04 ng/ml for

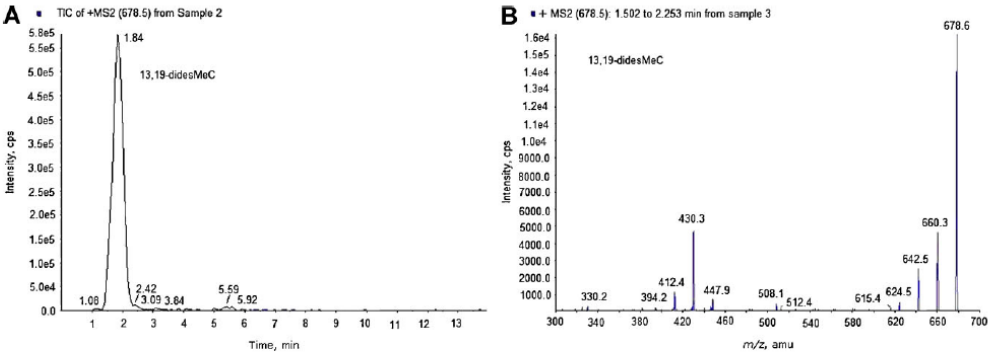


Fig. 4. (**A**) Chomatogram and (**B**) mass spectrum from the liquid chromatography-mass spectrometry 2 analysis of an *Alexandrium ostenfeldii* sample. The selected parent and product ion combinations were as follows: 13,19-didesMeC (*m/z* = 678.4; 660.4; 630.4). TIC = total ion chromatogram; cps = counts per second; MS = mass spectrometry; amu = mass shift.

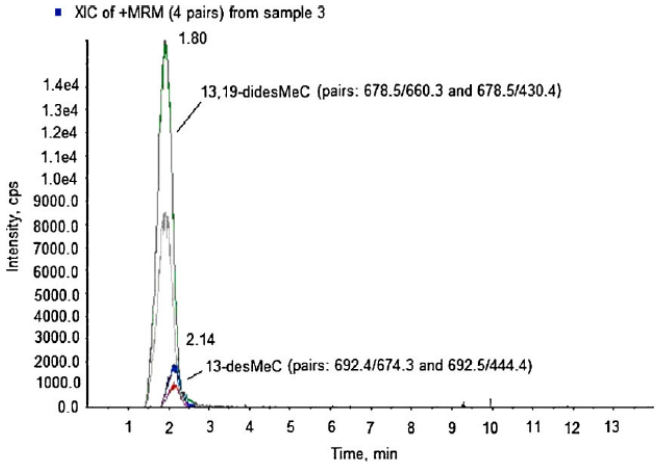


Fig. 5. Representative multiple reaction monitoring (MRM) chromatogram obtained from the crude phytoplankton extract on the triple-quadrupole mass spectrometer (QTRAP) from the sample 3. XIC = extracted ion chromatogram. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

13-desMeC and 4.35 ± 0.17 ng/ml for 13,19-didesMeC). Data obtained show high toxin values in cells cultured in seawater enriched with F/2 and Walne medium and low toxin content in the cultures grown with Algal. Moreover, the number of cells and the toxin per cell amount produced was also minor in Algal medium (Fig. 7). For F/2 medium, 6,245 ± 534 cells/ml were produced, as compared to 6,969 ± 993 cells/ml for Walne and 4,265 ± 378 cells/ml for Algal. Of the three media, Algal was clearly the least effective for cell and toxin production. Therefore, in the subsequent samples, only F/2 and Walne were employed.

Next, the effects of salinity on toxin content and composition were studied in two salinities (28 and 33‰) in 12 cultures grown at 19°C in F/2 and Walne medium, in 20 L of seawater and under constant light for 24 h. The information about toxin quantities and cells obtained are shown in Table 2. For 28‰ of salinity and Walne medium, 0.537 ± 0.057 pg/cell for 13-desMeC and 3.865 ± 0.500 pg/cell for 13,19-didesMeC were obtained. This is a proportion of 12.26 ± 0.32 % for 13-desMeC and 87.74 ± 0.32% for 13,19-didesMeC. Similar results were obtained for 28% of salinity and F/2 medium. However, for 33‰ of salinity and Walne medium, the amounts were 0.140 ± 0.014 pg/cell for 13-desMeC and 0.787 ± 0.125 pg/cell for 13,19-didesMeC. Equally low toxin amounts per cell were found for 33‰ of salinity and F/2 medium. The results evidence that salinity also affects the spirolide production per cell obtained. For both spirolides, this amount was four times less at 33‰.

Table 1. Spirolide toxin profile of CCMP1773 *Alexandrium ostenfeldii* cells from each sample

Cultures	Cells/ml	13-desMeC	13,19-didesMeC
1	3,258	0.03 pg/cell	8.5 pg/cell
2	2,860	0.02 pg/cell	2.1 pg/cell
3	2,630	0.06 pg/cell	6.2 pg/cell
4	6,890	0.04 pg/cell	1.5 pg/cell
5	4,068	0.07 pg/cell	2.7 pg/cell
6	5,922	0.04 pg/cell	1.3 pg/cell
7	1,900	0.03 pg/cell	0.9 pg/cell
8	4,532	0.08 pg/cell	2.5 pg/cell
9	4,876	0.05 pg/cell	1.4 pg/cell
10	4,256	0.04 pg/cell	1.3 pg/cell
11	3,998	0.03 pg/cell	1.3 pg/cell
12	3,944	0.07 pg/cell	1.9 pg/cell
13	4,186	0.03 pg/cell	1.3 pg/cell
Average values	4,101.5 ± 368.7	0.05 pg/cell ± 0.01	2.5 pg/cell ± 0.6

All cultures produced spirolides ranging from 0.9 to 8.5 pg/cell for 13,19-didesMeC (13,19-didesmethyl spirolide C) and from 0.02 to 0.08 pg/cell for 13-desMeC (13-desmethyl spirolide C). Cells were counted using a Sedgewick-Rafter camera (Lab Safety Supply).

Finally, to learn how the photoperiod can influence cell growth and toxin production, a new experiment was performed. Six new cultures of *A. ostenfeldii* were grown in 20 L of seawater with Walne and F/2 medium, at 19°C, 28‰ of salinity and subjected to a cycle of 14:10-h light:dark. The results are shown in Table 3, and were compared with the six previous experiments obtained at 28‰ without photoperiod (Table 2, cultures 1–6 A).

In general, the highest cell number was obtained in cultures treated with 14:10-h light:dark photoperiod for both F/2 and Walne medium. This value was nearly double the others. However, the least 13,19-didesMeC toxin amount per cell was found in cultures subjected to photocycle. The 13,19-didesMeC toxin amount was 1.4 times less in cultures treated with photoperiod for F/2 medium and 3.2 times less for Walne medium. On the other hand, the minor 13-desMeC amount per cell was obtained in cultures subjected to photoperiod and Walne medium, but 13-desMeC amount produced increased in cultures treated with F/2 and also with photoperiod. In summary, toxin proportion per cell found in cultures treated with F/2 medium, 28‰ of salinity, and photoperiod was different from those obtained in the previous experiments. The amount was 0.503 ± 0.031 pg/cell for 13-desMeC and 2.159 ± 0.028 pg/cell for 13,19-didesMeC, whereas the per-

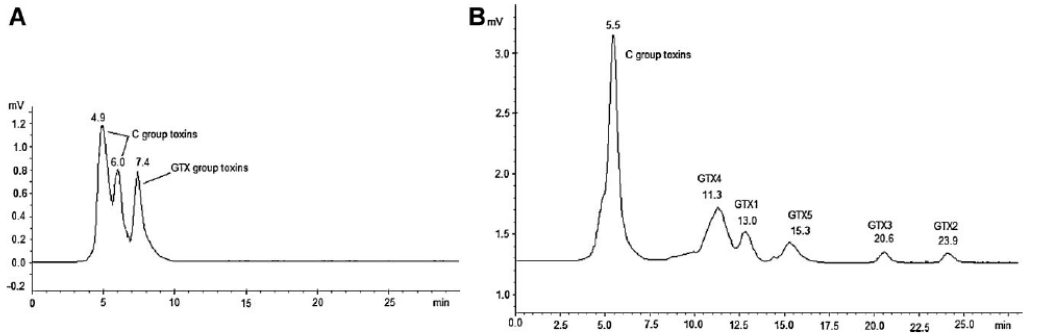


Fig. 6. Chromatograms obtained with a reversed phase column, Luna 4.6 × 250 mm, with mobile phase (**A**) 2 mM *n*-heptanesulfonic acid, 10 mM *o*-phosphoric acid, and acetonitrile 10%, and (**B**) 2 mM *n*-heptanesulfonic acid and 10 mM *o*-phosphoric acid. Both solutions were adjusted to pH 7.2 with ammonium hydroxide. GTX = gonyautoxin.

centage was 18.89 ± 1.10% for 13-desMeC and 81.11 ± 1.10% for 13,19-didesMeC.

These new cultures were analyzed by HPLC to determine the content of PSP toxins. The results showed that the amount of PSP produced was much lower than in the 13 initial cultures. To observe the differences found in toxin amounts more clearly, the three most representative profiles are shown in Figure 8. The higher amounts per cell for both spirolides were achieved when *A. ostenfeldii* dinoflagellate was grown in F/2 or Walne medium, under 24 h of light and salinity of 28‰ (Fig. 8B). These amounts were 3.87 ± 0.50 pg/cell of 13,19-didesMeC, which corresponds to 87.74 ± 0.32% from total spirolide produced and 0.537 ± 0.05 pg/cell for 13-desMeC that corresponds to the remaining 12.26 ± 0.32%. Nevertheless, a proportion of

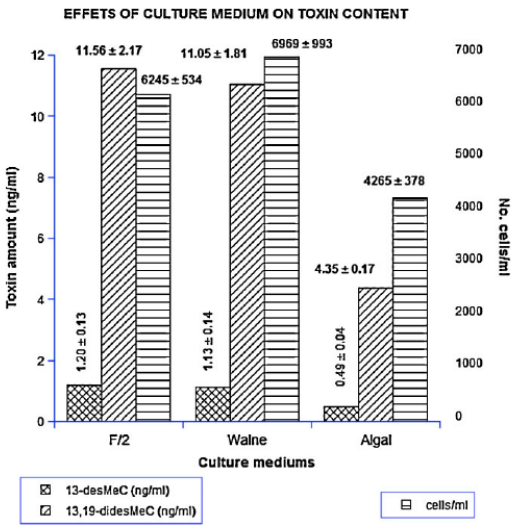


Fig. 7. Effects of F/2, Walne, and Algal medium on toxin profile and cell number. Three experiments were made in a water volume of 800 ml, salinity 28‰, and 24 h of light. Values are means of $n = 3$. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

97.60% ± 0.28 for 13,19-didesMeC was found in cells cultured in L1 medium with 14:10-h light:dark and salinity 33‰ (Fig. 8A). Finally, the highest proportion of 13-desMeC in cells was achieved when the *A. ostenfeldii* was cultured in F/2 medium 28‰ of salinity and 14:10-h light:dark photoperiod (Fig. 8C).

In summary, in the present study differences were found in the contents of spirolides produced by one strain of *A. ostenfeldii* growth under different environmental conditions. This percentage ranged from 2.40 to 18.89% for 13-desMeC and 97.60 to 81.11% for 13,19-didesMeC.

DISCUSSION

Worldwide, blooms of *A. ostenfeldii* tend to be aggregations at relatively low concentrations, usually minor to 10^3 cells/L. The highest cell concentration was found in eastern Siberia with 31×10^3 cells/L [14]. In water columns, the maximum density of cells is usually below the 4×10^3 cells/L found in Nova Scotia [15], 7.6×10^3 cells/L on the Danish coast [16], and 2.2×10^3 cells/L in Norwegian waters. The amount was enough to produce toxin that induced positive results with the mouse bioassay [11].

Several studies have demonstrated that *A. ostenfeldii* is the main producer of spirolides, although this dinoflagellate often produces PSP toxins. The production of either spirolides or PSP toxins by this dinoflagellate might be determined by salinity, temperature, or nutrients. In the present study, *A. ostenfeldii* was grown at controlled physical parameters of salinity, light, and temperature, and some cultures produced trace amounts of PSP toxins while other cultures did not produce them at all. The analyses to determinate the toxin profiles of this strain that originated from the Danish coast were carried out by means of LC-MS with a sensitive detection method. The sensitivity level was high with a detection limit greater than 17.5 nM. These results demonstrate that the strain CCMP1773 only produced 13-desMeC and 13,19-didesMeC under different environmental conditions. The samples analyzed in the present study did not have any mass in the region *m/z* 710.5 or *m/z* 712.5, which can be associated to spirolides E and F. This could be because both toxins are produced by hydrolysis of spirolides A and B in the

Table 2. Spirolide toxin amount, cell number, and toxin amount per cell produced by *Alexandrium ostenfeldii* dinoflagellate growth under different salinity regimes

Experiment	Culture medio	Salinity (‰)	No. cells/ml	13-desMeC (µg)	13,19-didesMeC (µg)	13-desMeC/cell (pg)	13,19-didesMe/cell (pg)
1 A	F/2	28	2,640	20.15	174.00	0.382	3.295
2 A	F/2	28	2,550	27.52	152.00	0.461	2.980
3 A	F/2	28	2,620	23.16	164.97	0.442	3.148
average			2,603 ± 27	23.84 ± 3.01	163.66 ± 6.38	0.428 ± 0.024	3.141 ± 0.091
4 A	Walne	28	3,000	28.55	197.8	0.476	3.219
5 A	Walne	28	2,650	25.63	186.93	0.484	3.527
6 A	Walne	28	2,310	30.09	224.06	0.651	4.850
average			2,653 ± 199	28.09 ± 1.31	202.93 ± 11.02	0.537 ± 0.057	3.865 ± 0.500
1 B	F/2	33	2,330	7.65	45.50	0.164	0.976
2 B	F/2	33	2,080	4.52	29.25	0.109	0.703
3 B	F/2	33	2,090	4.07	22.14	0.097	0.530
average			2,167 ± 82	5.41 ± 1.13	32.30 ± 6.91	0.123 ± 0.021	0.736 ± 0.130
4 B	Walne	33	2,350	5.40	25.75	0.113	0.541
5 B	Walne	33	2,710	7.94	51.24	0.146	0.945
6 B	Walne	33	2,110	6.78	37.00	0.161	0.876
average			2,390 ± 174	6.71 ± 0.73	38.00 ± 7.38	0.140 ± 0.014	0.787 ± 0.125

The culture volume was 20 L in the 12 experiments. 13,19-didesMeC (13,19-didesmethyl spirolide C); 13-desMeC (13-desmethyl spirolide C).

mollusk tissue [4,10], and they were never found in plankton samples. Nor were these toxins found in masses corresponding to spirolide B (*m/z* 694.5), spirolide C (*m/z* 706.5), spirolide D (*m/z* 708.5), 13-desMeD (*m/z* 694.5), 20-Me-G (*m/z* 706.5), or 27-OH-13,19-didesMeC (*m/z* 694.5). Spirolides A and G have the same molecular weight as spirolide 13-desMeC (691.5). The spirolide A molecule may present the same transitions found in spirolide 13-desMeC (*m/z* 678.5 and *m/z* 444.5), whereas spirolide G may only share the first transition (*m/z* 678.5). The retention times for these products match with spirolide standard 13-desMeC, and they do not appear in any other time, which dismissed the possibility of spirolides A and G's existence.

Some cultures of *Alexandrium* species were analyzed with differences in toxin composition depending on the region of origin. Several toxin profiles were found even in dinoflagellates from relatively close geographical origins [5]. This was probably due to the different characteristics of the coast. For instance, both Graves Shoal and Ship Harbour sites lay along the Eastern coast of Nova Scotia (Canada) within an interval of a few miles. Graves Shoal has open coastal waters, where dinoflagellates producing spirolides A and D were found, although spirolides A, B, 13-desMeC were found in Ship Harbour. Spirolides B, D, and 13-desMeD were also found in this region, although in a small amount. Equally, different spirolide toxin profiles had been found in samples obtained at the same site in the Gulf of Maine (USA) within a short lapse of just one month [8].

Different toxin production of indigenous *A. ostenfeldii* from the Danish coast have been described. Although strain k-0287 isolated from Limfjordan, Denmark, did not produce detectable amounts of spirolides [9], clones LF 37-CCMP1772 and LF-38-CCMP1773 produced different toxin profiles [3]. These profiles are different from the toxin profile showed in the present study. The LF-37 and LF-38 produced 13-desMeC and 13,19-didesMeC. Only the second culture produced spirolide G in a similar level to 13,19-didesMeC. The present study also found 13-desMeC and 13,19-didesMeC, but spirolide G did not appear in our samples.

There are few studies on the effects of environmental factors in the production of toxins in the *Alexandrium ostenfeldii* species [14,17]. The relation between environmental and genetic factors and toxin production is highly complex among the dinoflagellates. The few theories suggest that the quantity of spirolide toxins synthesized by *A. ostenfeldii* is increased with cell concentration, but cell spirolide quota and toxin composition do not differ in response to variations in environmental regime [17]. Others show that the total toxin present in batch cultures is affected by some environmental conditions such as intensity of light and photoperiod [16]. But all of them support the theories that the composition of the toxins, that is, the profile produced remains constant despite environmental

Table 3. Spirolide toxin amount, cell number, and toxin amount per cell produced by *Alexandrium ostenfeldii* dinoflagellate growth under 28‰; F/2 (F/2 medium) and W (Walne) medium and photoperiod of 14:10 h light:dark

Experiment	Culture medio	Salinity (‰)	No. cells/ml	13-desMeC (µg)	13,19-didesMeC (µg)	13-desMeC/cell (pg)	13,19-didesMeC/cell (pg)
1 C	F/2	28	3,520	39.39	148.2	0.559	2.105
2 C	F/2	28	3,540	35.30	155.8	0.498	2.201
3 C	F/2	28	6,280	56.87	272.6	0.452	2.170
average			4,447 ± 916	43.85 ± 6.61	192.2 ± 40.26	0.503 ± 0.031	2.159 ± 0.028
4 C	Walne	28	3,460	22.62	78.75	0.327	1.138
5 C	Walne	28	2,060	14.57	59.94	0.354	1.455
6 C	Walne	28	8,820	37.79	184.8	0.214	1.048
average			4,780 ± 2,060	24.99 ± 6.81	107.83 ± 38.87	0.298 ± 0.043	1.214 ± 0.123

The culture volume was 20 L in the six experiments. 13,19-didesMeC (13,19-didesmethyl spirolide C); 13-desMeC (13-didesmethyl spirolide C).

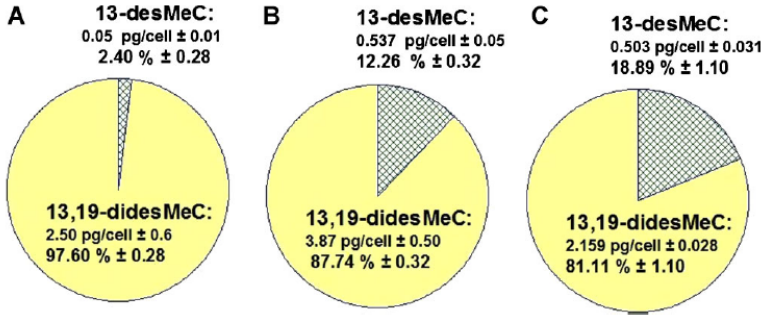


Fig. 8. Spirolide toxin profile from the *Alexandrium ostenfeldii* strain CCMP177 obtained under the following conditions: (**A**) salinity 33‰, L1 medium with daylight lamps (14:10 h light:dark) $n = 13$, and (**B**) salinity 28‰, F/2 and W medium, 24 h of light $n = 3$. (**C**) salinity 28‰, F/2 medium and photoperiod $n = 3$. 13-desMeC = 13-desmethyl spirolide C; 13,19-didesMeC = 13,19-didesmethyl spirolide C. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

changes [16,17]. We think that the type of spirolide produced by *A. ostenfeldii* is linked to a genetic origin, but their proportions can vary considerably depending on the culture mode. In the present study, the dinoflagellate synthesizes more spirolide per cell in a salinity of 28 than 33‰. A larger amount of 13,19-desMeC was observed in samples grown with 24 h of light compared to the cultures subject to photoperiod, whereas the highest proportion of 13-desMeC in cells was achieved in F/2 medium, 28‰ of salinity, and photoperiod. Contrary to some theories [17], the toxin amount was not increased with cell concentration when Walne medium, 28‰ of salinity, and photoperiod were employed. We propose to culture the dinoflagellate in F/2 and Walne medium, 28‰ of salinity, and 24 h of light to obtain a high toxin quota per cell for both spirolides. To achieve a greater proportion of 13,19-didesMeC with respect to the other one, L1 medium with salinity of 33‰ and photoperiod (14:10-h light:dark) must be employed. On the contrary, the highest proportion of 13-desMeC in cells was obtained when the *A. ostenfeldii* was cultured in F/2 medium, 28‰ of salinity, and also a 14:10-h light:dark photoperiod.

The present study shows the first evidence that external environmental factors can influence the toxin profile produced by *A. ostenfeldii* dinoflagellate. This percentage ranged from 2.40 to 18.89% for 13-desMeC and 81.11 to 97.60% for 13,19-didesMeC. These varieties found in the toxin profile are significant percentages if it is taken into account that many authors argue that the spirolide toxin profile of some *Alexandrium* species are independent of culture conditions [16,17]. The present study provides information on toxin profiles from a Danish strain that grew in batches under different controlled conditions, proposes some optimum conditions for growing the dinoflagellate and obtaining high quantities of spirolide per cell never found before in species of *A. ostenfeldii*.

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I.2. Nuevo protocolo para obtener SPXs a partir de cultivos de *A. ostenfeldii* con gran recuperación y pureza.

Resumen

Para cuantificar toxinas mediante métodos de detección químicos es de suma importancia la utilización de estándares para cada una de ellas. Muchas de estas toxinas no están disponibles como patrones o son difíciles de obtener con gran pureza. La importancia de este trabajo es el diseño de un procedimiento completo para extraer grandes cantidades de SPXs y purificarlos a gran escala a partir de los cultivos de la cepa de *A. ostenfeldii*. Para la obtención de los compuestos puros se puso a punto un protocolo de extracción y limpieza de las muestras con distintos disolventes y posterior purificación en HPLC en escala preparativa. Para proceder a la extracción de las toxinas, separamos las células del agua por filtración y se realizaron varias extracciones con disolventes orgánicos y varias particiones líquido-líquido. En este proceso se consiguió separar los SPXs de las toxinas PSP con un alto porcentaje de recuperación. Para limpiar las muestras se realizaron varias cromatografías de permeabilización de gel, empleando varias columnas de Sephadex LH-20, cargando la muestra con metanol y eluyendo con acetona. Finalmente, la purificación de ambas toxinas (13-desMeC y 13-didesMeC) se realizó en HPLC preparativo acoplado a un detector MS. La pureza y cantidad de ambas toxinas en cada paso se detectó por LC-MS/MS. Ambos compuestos se han conseguido en grandes cantidades y con una pureza del 97% para 13-desMeC y del 99% para el 13,19-didesMeC. El protocolo demostró ser un método eficaz para obtener SPXs con alta pureza, repetitividad, estabilidad y recuperación.

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New protocol to obtain spirolides from *Alexandrium ostenfeldii* cultures with high recovery and purity

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ABSTRACT: The aim of this work was to develop a method to purify large amounts of spirolide toxins from cultures of *Alexandrium ostenfeldii*. The dinoflagellates grew in batches under controlled conditions of salinity, light and temperature. Analysis of the cultures demonstrated the existence of neurotoxins associated with paralytic shellfish poisoning toxins and two spirolides, 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C. The protocol designed presents several stages of extraction, separation between spirolides and paralytic shellfish poisoning toxins, and cleanup in solid-phase extraction. Finally, the purification of spirolides was conducted by a preparative high-performance liquid chromatography system coupled to a mass spectrometer detector. The purity and the amount of both toxins in each step was monitored by analytical liquid chromatographic–mass spectrometry. Large amounts of 13-desMeC, 97% pure, and 13,19-didesMeC, 99% pure, were obtained. A novel and efficient method to separate and purify spirolide toxins from large amounts of phytoplankton is provided. The protocol proposed shows, for the first time, a complete and detailed methodology to separate and purify spirolide toxins with high purity, recovery, repeatability and stability. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: spirolides; purification, 13-desmethyl spirolide C; 13,19-didesmethyl spirolide C; LC-MS; analytical and preparative methodology

Introduction

Spirolides are macrocyclic polyether compounds that belong to the cyclic imine group. This group includes other lipophilic compounds such as gymnodimine, pinnatoxins, prorocentrolide, pteriatoxins and espiro-prorocentrimine (MacKinnon *et al.*, 2004, 2006a, b). Spirolides are the most common cyclical imine toxins due to their widespread global distribution. They were first identified in extracts of the digestive glands of mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*) from the Atlantic coast of Nova Scotia, Canada, in 1991 (Meilert and Brimble, 2006). That year, routine toxin monitoring of bivalve molluscs in this location revealed a toxic response in mice after intraperitoneal injections of shellfish extracts. The clinical manifestations included abdominal muscle spasms, piloerection, ataxia, tail whipping and, immediately prior to death, neurological symptoms, comprising convulsions and cramps. Chemical investigations demonstrated that the toxicity was due to a new class of toxins which were called spirolides.

Spirolide toxins have now been detected in shellfish from a number of other locations around the world. In Europe, these toxins were found in Spain (Villar Gonzalez *et al.*, 2006), Italy (Ciminiello *et al.*, 2006), Scotland (John *et al.*, 2003), Norway (Aasen *et al.*, 2005) and Denmark (MacKinnon *et al.*, 2006b). The dinoflagellate identified as the producer of spirolides, in all these coasts, was *Alexandrium ostenfeldii*. Many studies report this organism to be the main origin of spirolides, although this dinoflagellate may produce toxins associated with paralytic shellfish poisoning (PSP).

There have been no reports of toxic effects in humans due to ingestion of spirolides. However, general symptoms such as tachycardia and gastric distress were reported following contaminated shellfish consumption from Nova Scotia during the spring and summer of 1991, when spirolides were detected in mollusc from this region (Richard *et al.*, 2001; Sleno and Volmer, 2005). Although the mechanism of action of spirolides in cells are not yet well understood, it has been suggested that the muscarinic acetylcholine receptors might be implicated in its mode of action (Gill *et al.*, 2003).

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) methods were used to achieve the structure elucidation of spirolides (Aasen *et al.*, 2005; Ciminiello *et al.*, 2006, 2007; Hu *et al.*,

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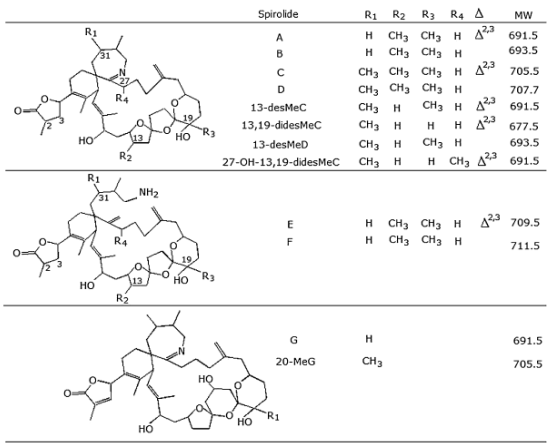
Abbreviations used: dcGTX, decarbamoylgonyautoxins; dcSTX, decarbamoylsaxitoxin; GTX, gonyautoxins; NEO, neosaxitoxin; PSP, paralytic shellfish poisoning; STX, saxitoxin; TFA, trifluoroacetic acid.



Spirolide	R_1	R_2	R_3	R_4	Δ	MW
A	H	CH_3	CH_3	H	$\Delta^{2,3}$	691.5
B	H	CH_3	CH_3	H		693.5
C	CH_3	CH_3	CH_3	H	$\Delta^{2,3}$	705.5
D	CH_3	CH_3	CH_3	H		707.7
13-desMeC	CH_3	H	CH_3	H	$\Delta^{2,3}$	691.5
13,19-didesMeC	CH_3	H	H	H	$\Delta^{2,3}$	677.5
13-desMeD	CH_3	H	CH_3	H		693.5
27-OH-13,19-didesMeC	CH_3	H	H	CH_3	$\Delta^{2,3}$	691.5
E	H	CH_3	CH_3	H	$\Delta^{2,3}$	709.5
F	H	CH_3	CH_3	H		711.5
G	H					691.5
20-MeG	CH_3					705.5

Figure 1. Structural variants and molecular weight (MW) of known spirolides isolated from shellfish and plankton. Compounds with 2,3 have a double bond between carbons 2 and 3.

2001; MacKinnon *et al.*, 2006b; Sleno and Volmer, 2005; Sleno *et al.*, 2004b). The structures and molecular weight (MW) of common known spirolides are presented in Fig. 1. Chemistry data obtained reveal that these toxins exist in three groups. In the first group, the toxins have a 6:5:5-polyether ring system in addition to the heptacyclic imine ring. This group includes spirolides A, B, C, D, some des-methyl derivatives of spirolides C and D, namely 13-desmethyl spirolide C (13-desMeC), 13,19-didesmethyl spirolide C (13,19-didesMeC) and 13-desmethyl spirolide D (13-desMeD) and the hydroxilated analog 27-hydroxy-13,19-didesmethyl spirolide C (27-OH-13,19-didesMeC). The second group has the same polyether ring system, but not cyclic moiety. They were designated with the letters E and F and they are shellfish metabolites in which the cyclic imines of spirolides A and B have been opened to a keto amine. Indeed, the imine group is typically considered to be a reactive functional group sensitive to hydrolysis (Pelc and Zakarian, 2005); however the seven-member cyclic imine in spirolides C and D has been shown to be completely stable to hydrolysis upon treatment with aqueous acids (Hu *et al.*, 2001; Pelc and Zakarian, 2005). Finally, the third group composes spirolide G and their variant 20-methylG (20-MeG). Both have the imine ring intact and contain an unusual 5:5:6-trispiroketal ring system never observed before in other marine toxin groups.

The spirolides are soluble in organic solvents such as methanol and acetone; therefore they are easily extracted from digestive glands and meat. The LD_{50} of a mixture of spirolides, comprising mostly 13-desMeC, was determined to be 40 μg/kg after intraperitoneal injection in mice and 1 mg/kg after oral administration (Rex, 2008; Richard *et al.*, 2001). The intact cyclic imine ring is elemental for toxicity. When the imine group is reduced, as in spirolides E and F, the toxicity is greatly decreased (Falk *et al.*, 2001; Hu *et al.*, 1996; Rex, 2008).

High-performance liquid chromatography (HPLC) is widely used in the separation, purification and isolation of toxins. Its application to the identification and quantification of these compounds renders high sensitivity, precision and reproducibility, giving information on the qualitative and quantitative composition. The aim of this work is to develop a method to purify 13-desMeC and 13,19-didesMeC from phytoplankton samples, using HPLC coupled to MS detection. The toxin profile of this strain of *A. ostenfeldii* dinoflagellates has been previously evaluated. These cells produce an uneven proportion of two spirolides, 13-desMeC and 13,19-didesMeC, and trace amounts of toxins belonging to the PSP group (GTX and C toxins).

Materials and Methods

Chemicals and Reagents

All solvents employed in this work were HPLC or analytical grade and the water was distilled and passed through a water purification system from Arium 611 Sartorius (Goettingen, Germany). Acetonitrile, dichloromethane, methanol, trifluoroacetic acid (TFA), ethanol, periodic acid and acetic acid 0.03 M were purchased from Panreac Quimica S.A. (Barcelona, Spain). Formic acid and ammonium formate were from Merck (Darmstadt, Germany). Sephadex LH-20, sodium phosphate, *n*-heptanesulfonic acid and *o*-phosphoric acid were obtained from Sigma-Aldrich (Seelze, Germany). Standard solution of 13-desmethyl spirolide C (13-desMeC) was purchased from the Institute for Marine Biosciences, National Research Council of Canada. Each ampoule contained 0.5 mL of solution with 10.2 μM 13-desMeC in methanol with 0.05% (v/v) TFA. 13,19-Didesmethyl C (13,19-didesMeC) standard was purchased from Laboratios CIFGA S.A. (Lugo, Spain). The ampoules contained 0.5 mL of solution with 14.8 μM 13,19-didesMeC in methanol with 0.05% (v/v) TFA.

Harvest of *A. ostenfeldii* and Samples Preparation

Alexandrium ostenfeldii dinoflagellates were isolated from Limfjorden (Denmark), strain CCMP1773. The culture came from The Provasoli Guillard National Center for Culture of Marine

Phytoplankton. The dinoflagellate were inoculated and maintained in 500 mL Erlenmeyer flasks under controlled conditions prior to passing the inocula to 20 or 40 L bags. Cells were counted using an Sedgewick-Rafter camera (Lab Safety Supply, Janesville, WI, USA). The cultures grew in seawater enriched with two culture media: F/2 and Walne (W) at 28‰ of salinity. The culture bags were shaken gently by hand and the growth occurred at 19°C. Twenty-four cultures were used to perform the toxin extraction and the toxin profile of this strain is 13-desMeC, 13,19-didesMeC and PSP toxins.

Equipment

- Evaporators: rotary evaporator R-200 from Büchi (Flawil, Switzerland), miVac centrifugal concentrator from Genevac (Ipswich, UK) and Büchi® Syncore Systems for Parallel Evaporation from Büchi.
- Vacuum system: a VAC Elut SPS 24, from Varian (Palo Alto, CA, USA) was employed to force the passage of solvents in the Sephadex LH-20 columns.
- LC-MS equipment: a combination of HPLC plus mass detector. The HPLC system, from Shimadzu (Kyoto, Japan), consisted of two pumps (LC-10ADvp), sn autoinjector (SIL-10ADvp) with refrigerated rack, a degasser (DGU-14A), a column oven (CTO-10ACvp) and a system controller (SCL-10Avp). This system was coupled to a QTRAP LC/MS/MS system from Applied Biosystems (Bedford, MA, USA), consisting of a hybrid quadrupole–linear ion trap mass spectrometer equipped with an API fitted with an ESI source. A nitrogen generator NM20ZA from Peak Scientific (Billerica, MA, USA) was used.
- HPLC system: for analytical HPLC a Shimadzu system equipped with two LC-10ADvp pumps, one LC-6A pump, a SIL-10ADvp automatic injector (10 µL injection loop), a SCL-10Avp system controller and one RF535 fluorescence detector, set at 340 nm excitation and 410 nm emission wavelengths, from Shimadzu was used. Data were analyzed with Class-vp software.
- LC-MS preparative and analytical system: the preparative and analytical system used for purification was from Waters (USA), and consisted of a pump (Delta 600), sample manager (2767), fraction collector (III) and controller (600), coupled to a column oven (CTO-10ACvp) from Shimadzu (Japan). This system was coupled to a mass spectrometer (Micromass Quattro micro API, from Waters). The system had another HPLC pump 515 and a flow splitter 1:100 from Waters (Ireland) to split the sample between the mass spectrometer detector and the collector.

Liquid Chromatography–Mass Spectrometry Analysis

All aliquots saved in the process of extraction and purification, were analyzed by means of LC-MS to determine the spirolide content and percentage of recovery in each step. The column used for separations was a 2 × 50 mm BDS-Hypersil-C8 analytical column with a particle size of 3 µm and a 10 × 2.1 mm guard cartridge from Thermo (Waltham, MA, USA). The temperature was set at 25°C. The mobile phase consisted of two components: water (A) and acetonitrile–water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 30–90% B for 8 min, then, 90% B and 10% A were held for 3 min and reduced afterwards to 30% B over 0.5 min. Then, they were held again for 2.5 min until the next run. The mobile phase flow rate was 0.2 mL/min and the injection volume was 5 µL. Collision-induced dissociation (CID) in the ion-trap MS was carried out on the protonated molecule, $[M + H]^+$, for each toxin. For each standard, a good eight-point calibration line among the range 12.5–1000 ng/mL was obtained ($R^2 = 0.99$). The ESI source of QTRAP was operated with the following optimized values of source-dependent parameters: Curtain gas™, 15 psi; collision-activated dissociation gas (CAD), 6 psi; ionspray voltage, 4000 V; temperature, 450°C; gas 1, 50 psi; and gas 2, 50 psi. Nitrogen was used as curtain and CAD gas. The multiple reaction monitoring (MRM) experiments were realized by selecting the following groups of transitions: 692.5 > 674.5 and 692.5 > 444.5 (for 13-desMeC) and 678.5 > 660.5 and 678.5 > 430.5 (for 13,19-didesMeC).

Analysis of PSP Toxins

PSP toxins analyses were done by HPLC with post-column derivatization and fluorescence detection, based on a variation of the method of Oshima (Alfonso *et al.*, 1993). The separation was carried out on a reversed-phase column (Luna 4.6 × 250 mm, 5 µm). Two types of mobile phases were used to detect different groups of PSP toxins. *n*-Heptanesulfonic acid, 2 mM, *o*-phosphoric acid, 10 mM, and acetonitrile 10% were applied to detect saxitoxin (STX) and its analogs, neosaxitoxin (NEO) and decarbamoylsaxitoxin (dcSTX). Alternatively, 2 mM *n*-heptanesulfonic acid and 10 mM *o*-phosphoric acid were used to detect gonyautoxins (GTX) and its analog, decarbamoylgonyautoxins (dcGTXs). Both solutions were adjusted to pH 7.2 with ammonium hydroxide. Samples were evaporated to dryness, the residues were dissolved in the equivalent volume of acetic acid 0.03 M and passed through 0.45 µm filters (Millipore Ultrafree-MC centrifugal filter units) to be analyzed by HPLC. Post-column oxidation was carried out by a solution composed of 7 mM periodic acid and 50 mM sodium phosphate, adjusted to pH 9. This solution was mixed with toxins eluted from the column in a T made from Teflon. The resulting mixture was heated while passing through a tube immersed in a water bath at 70°C. Subsequently, this mix was acidified in another T with acetic acid to obtain pH 5–7. The resulting solution was measured in the fluorescence detector.

LC Preparative and Analytical System Coupled to a Mass Spectrometer to Purify

LC separation. Separations were performed by isocratic chromatography, acetonitrile–water plus 0.1% TFA. The proportions of these solvents were modified in analytical method to obtain an optimal separation. The analytical chromatography was realized with a Vydac 201TP52 C_{18}, 250 × 2.1 mm i.d., 5 µm column at a flow rate of 0.2 mL/min and the preparative method employed a Vydac 201TP510 C_{18}, 250 × 10 mm i.d., 5 µm, column at a flow rate of 5 mL/min. The temperature was 25°C in both cases.

MS detection. MS parameters were adjusted to obtain a signal of maximum intensity and stability. For the MS parameter optimization, the working solution (2 µg/mL 13-desMeC) was continuously infused in the electrospray source at a 10 µL/min flow rate with a syringe pump. The mass spectrometer was operated in the positive ion mode using SIR (selecting ion recording) with the following parameters: cone gas, 50 V; capillary voltage, 3 kV; source temperature, 100°C; desolvation temperature, 200°C; desolvation gas flow, 250 L/h; entrance, 50 eV; collision, 1 eV; exit,

50 eV; multipler, 500 V. Helium and nitrogen were used as collision and drying gases, respectively.

Results

Twenty-four cultures of *A. ostenfeldii* were used to extract and purify the spirolides toxins, 13-desMeC and 13,19-didesMeC. The cultures were grown in a volume of 20 or 40 L within a few months for approximately 30 days. Cells were collected by gravity filtration through a 0.20 µm mesh. The filtrated cells were suspended in methanol with a ratio of three volumes of solvent per cell volume, broken by ultrasound while cooling in ice bath and stored at −20°C prior to extraction. Then, these extracts were centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was removed and fresh methanol was added over the pellet. This step was repeated three times. The supernatants were collected and the amount of toxins was quantified by HPLC and LC-MS. In these extracts trace amounts of PSP toxins, 600 ± 43 µg of 13-desMeC and 4680 ± 359 µg of 13,19-didesMeC, were obtained. Figure 2 shows the LC-MS chromatogram of 13-desMeC, eluted at 6.14 min retention time, and 13,19-didesMeC, eluted at 4.90 min.

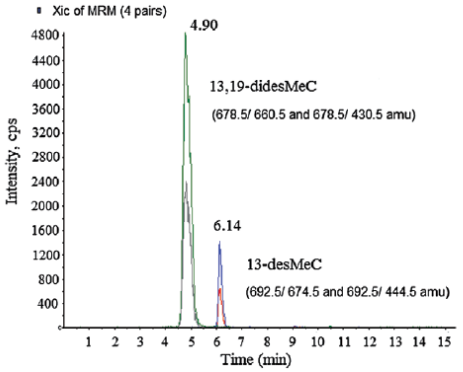


Figure 2. Chromatogram in positive mode from a methanol extract (QTRAP instrument). The sample was obtained after extraction of *A. ostenfeldii* cultures with methanol.

After the extraction procedure, a partition with organic dissolvent was performed in order to separate the PSP group and to eliminate fat and hydrophilic contaminants. The methanol extract was concentrated and the residue was dissolved in water. The suspended extract was partitioned three times against dichloromethane. After this step three organic colored extracts were obtained, which were mixed, dried and analyzed. The aqueous phase was also removed from the funnels and analyzed. In these partitions, a dark layer was situated between organic and aqueous phase. The analysis of dichloromethane and aqueous phases was done by HPLC for PSP toxins and by LC-MS for spirolide toxins. In the organic layer 560 ± 35 µg of 13-desMeC and 4687 ± 472 µg of 13,19-didesMeC were found, while the PSP toxins were detected in the water phase. In summary after the dichloromethane–water partition, the two main toxin groups were separated and contaminants and fat were removed.

The next step in the purification protocol was a solid-phase extraction (SPE). This process is necessary in order to eliminate interfering contaminants in the purification method. Several articles describe the use of Sephadex-LH 20 for cleaning samples prior to HPLC. In all these articles, the sample was loaded into the sephadex column dissolved in dichloromethane and then eluted with methanol (Gill *et al.*, 2003; Hu *et al.*, 2001; MacKinnon *et al.*, 2006b). In the present study, several cleanup tests were carried out in 2 mL polypropylene columns (Supelco). Several toxin concentration, solvents, sample volumes and sample natures were used in each case. All these experiments are described in Tables 1 and 2 (experiments 1–16). The tables shown the amount and volume of toxin loaded in the column, the eluting solvent and the amount of toxin recovered, expressed as percentage, after cleanup. Data from Table 1 were obtained when the toxin was diluted in dichloromethane. Results from experiments 1–6 and 9–11 shown that methanol, ethanol and acetonitrile 90% were not good eluents when the toxin was loaded with dichloromethane since recoveries were around 50%. However, full toxin recovery, more than 89%, was achieved when the sample was eluted with acetonitrile 50% or acetone (experiments 7, 8 and 12 to 14). Table 2 shows two experiments done with these two last solvents as eluents but where the sample was diluted in methanol (instead

Table 1. SPE results obtained in the cleaning procedure with samples loaded in dichloromethane. In each experiment different eluting solvent or sample volume was used. The fractions obtained after cleaning were dried, diluted in methanol and analyzed by LC-MS to detect the spirolide amount. This amount is expressed as a percentage of toxins recovered in each experiment

Experiment (no.)	Sample volume (µL)	Spirolide (ng/mL)	Eluting dissolvent	Recovery total (%)
1	1000	187.5	Methanol	50.2
2	900	222	Methanol	49
3	900	222	Ethanol	57
4	900	222	Acetonitrile 90%	53.5
5	800	223	Methanol	36
6	800	223	Ethanol	40
7	800	223	Acetonitrile 50%	**100**
8	800	223	Acetone	89
9	800	93.3	Acetonitrile 90%	78
10	400	390	Acetonitrile 90%	0
11	800	195	Acetonitrile 90%	54
12	800	142.5	Acetone	**100**
13	400	390	Acetone	52
14	800	195	Acetone	77

Table 2. SPE results obtained in the cleaning procedure with samples loaded in methanol. The fractions obtained after cleaning were dried, diluted in methanol and analyzed by LC-MS to detect the spirolide amount. This amount is expressed as a percentage of toxins recovered in each experiment

Experiment (no.)	Sample volume (µL)	Spirolide (ng/mL)	Eluting dissolvent	Recovery total (%)
15	800	265	Acetonitrile 50%	100
16	800	195	Acetone	98

dichloromethane). In both cases, the recovery was complete (100 and 98% respectively). Based on these results, the option of loading the sample diluted in methanol and then using acetone to elute was chosen to clean the samples. In these conditions the whole sample was diluted in methanol, loaded in 25 mL polypropylene columns and eluted with acetone. The fractions obtained were combined, evaporated to dryness and dissolved in methanol. In this step less than 2% of toxin was released.

The last step in the purification was the separation of 13-desMeC from 13,19-didesMeC by liquid chromatography with mass detection. To obtain a good separation and recovery, some factors were first tested by analytical chromatography and then transferred to a preparative method. Different proportions of acetonitrile–water with 0.1% TFA are usually used to separate spirolides. In the present paper three mobile phases were checked: 40:60 (v/v), 35:65 (v/v) and 30:70 (v/v) acetonitrile–water. Three injections were performed with the same conditions (temperature, flow and injection volume, 25°C, 0.2 mL/min and 20 µL respectively). As shown in Fig. 3, the retention times and the separation between toxins were the same with all three mobile phases; however the highest intensity, which means highest recovery, was obtained with 30:70 (v/v) acetonitrile–water plus 0.1% TFA (Fig. 3C). From these results, this mobile phase was selected. Then these parameters were adjusted to the preparative chromatography where the mass-triggered mode was selected as fraction collector. This mode was considered more efficient because the collected fractions can be accurately differentiated by their *m/z* values. The masses selected for the collection of compounds 13-desMeC and 13,19-didesMeC were the parent ions 692.5 *m/z* and 678.5 *m/z*, respectively. The methanol samples from SPE were passed through a 0.45 µm filter and then injected (volume 500 or 750 µL). The peaks of interest were well resolved under the isocratic conditions 30:70 (v/v) acetonitrile–water and flow 5 mL/min. In these conditions the retention times were 9.5 min for 13,19-didesMeC and 13.5 min for 13-desMeC with a high repeatability between different injections. Figure 4(A) shows a representative chromatogram of a preparative injection with the fractions obtained. As the figure shows, the two toxin peaks were separated and five fractions in each injection were separated and collected. Each fraction was concentrated to dryness, dissolved in 500–750 µL methanol with 0.05% TFA and evaluated by analytical LC-MS. Figure 4(B) illustrates the amount of toxin and the percentage of purity in each fraction. As the figure shows, most of 13,19-didesMeC was eluted in fractions 3 and 4 with purity higher than 99%, while most of 13-desMeC was collected in fraction 5, with 96.2 % purity. In

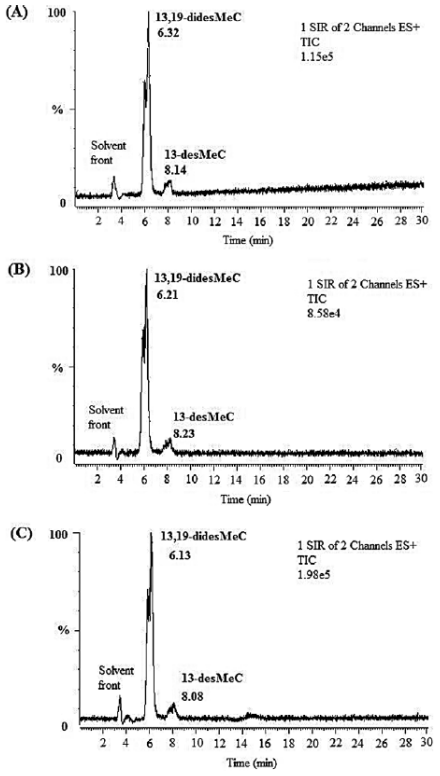


Figure 3. Analytical chromatograms in mode positive SIR (Waters analytic and preparative LC-MS). The mobile phase was composed of acetonitrile–water (A) 40:60, (B) 35:65 and (C) 30:70 with 0.1% TFA. The column temperature was 25°C and the flow selected was 0.2 mL/min.

these experiments the recovery of toxin after each injection was also evaluated. As shown in Table 3, in five experiments the recoveries were around 81.2 ± 1.3%, which means less than 18% of toxin was released in each injection, with therefore high recoveries in terms of purification.

From these results, the next step was to determine the maximum amount of toxin per injection based on the purity and recovery obtained for both toxins. The results are shown in Table 4. First, 2.6 ± 0.1 µg of 13-desMeC and 13.2 ± 0.4 µg of 13,19-didesMeC were injected (test 1). As the table shows, high purity and recovery were obtained. Then the toxin amount by injection was increased to 8.2 ± 0.2 µg of 13-desMeC and 54.8 ± 0.9 µg of 13,19-didesMeC in test 2. Purity decreased to 92.0 ± 0.8% for 13-desMeC because the two toxins eluted closer. Next, the same toxin amount was injected but filling the collection tubes only 10% in order to achieve the overlap of two toxins in low volume (test 3). In this case, the recovery decreased to 60%. Finally, in test 4, 3.36 ± 0.6 µg of 13-desMeC and 28 ± 0.9 µg of 13,19-didesMeC

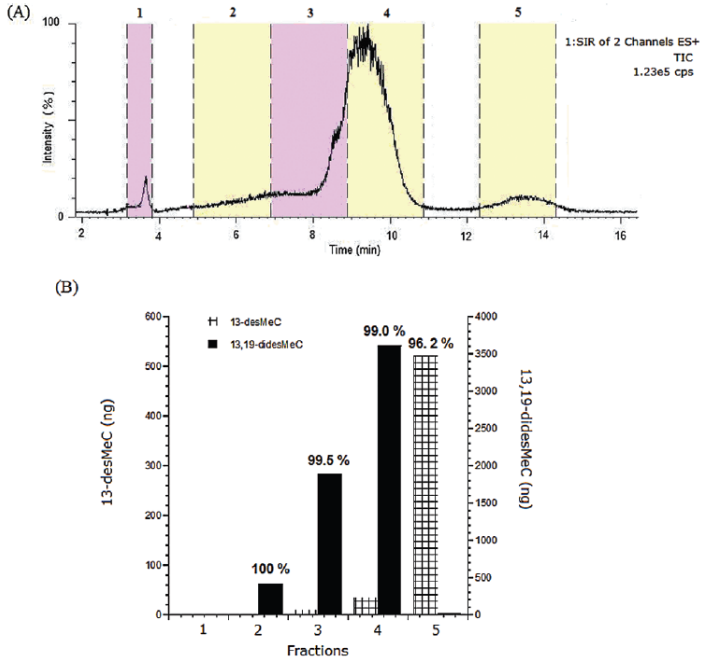


Figure 4. Preparative chromatogram and fractions after sample injection. (A) Total ion chromatogram (TIC) in mode positive SIR during fraction collection in preparative method. Five fractions were collected and the high intensity obtained was 1.23×10^5 cps. The minimum collection intensity was 5500 cps and the injection volume 500 µL. (B) Amount of toxins detected in the each fraction collected. The purity percentage of each toxin is indicated on the top of each column.

Table 3. Recovery results from five sample injections by preparative LC-MS. The column used was a Vydac C_{18} TP (250 × 10 mm) and oven temperature was set at 25°C. The phase mobile conditions were: 30:70 (v/v) acetonitrile–water with 0.1% TFA and 5 mL/min. The spirolide toxin recovery was 81.2 ± 1.3%

Injection	Injection volume (µL)	Sample concentration (µg/mL)	Recovery (%)
a	500	7.0	86.0 %
b	750	7.0	79.0 %
c	750	5.8	78.5 %
d	500	5.8	81.9 %
e	500	16.2	80.5 %

were injected. These quantities ensured an 80% of recovery for both toxins and a good purity. Thus, a total of 1185 ± 0.090 µg 13,19-didesMeC and 117 ± 0.020 µg 13-desMeC were purified.

In order to improve the purity of 13-desMeC, the extract of 92.0 ± 0.8% of purity obtained in test 2 was passed thought preparative column again. The resulting chromatogram obtained is shown in Fig. 5(A). The purity achieved was 99.9 ± 0.5% and the recovery was 81.1 ± 0.8%. Figure 5(B) represents the 13,19-didesMeC compound of 98.7 ± 2.0% purity obtained in test 4.

Hence, from these results a purification protocol for spirolides from dinoflagellates cultures is shown in Fig. 6. All necessary steps in order to obtain these compounds with high purity, recovery, repeatability and stability are provided.

Discussion

Many efforts have been made to determine algal toxins with chemical methods (Christian and Luckas, 2008). Bioassays are common methods for the determination of marine toxins, but they are not completely satisfactory, due to their low sensitivity and poor selectivity. Analytical methods for spirolide toxins are essential to avoid the human food poisoning caused by their consumption, but research has been restricted by a shortage of the pure compounds. This toxins can be obtained in limited amounts from contaminated shellfish; therefore, it is necessary develop a large-scale method for extraction of the toxins from phytoplankton samples (Rundberget *et al.*, 2007).

Spirolides are lipophilic toxins; therefore they are removed easily with organic solvent such as methanol, ethanol or acetone. Some authors perform two extractions with 80% aqueous

methanol from mussel digestive glands (Aasen *et al.*, 2005, 2006) and this solution was also used for removing the toxins from *A. ostenfeldii* cells (Aasen *et al.*, 2006). In another paper, spirolides were first extracted from cells with the same aqueous methanol solution and then two other extractions with methanol (Ciminiello *et al.*, 2006). In the present paper, the toxins were extracted from cultures that grow in seawater, and some water was also retained with cells in the filtration process. Therefore, the phytoplankton samples were hydrated and consequently we performed four methanol 100% extractions. To ensure that the extraction of the toxins was complete, many of the purification steps needed to be done repetitively. We propose four extractions with methanol to achieve this objective.

The methanolic extracts were pooled and concentrated at $t^a <$ 25°C. The temperature is an important feature because several marine toxins are destroyed by heat. The dry residue was taken up in water and partitioned three times with dichloromethane. Some methods that purify spirolides from digestive glands of mussel (spirolide G) (Aasen *et al.*, 2005) and from phytoplankton cells (13-desMeC) (Sleno *et al.*, 2004a) used a first partition with

Table 4. Recovery and purity obtained from several injected quantities, in a volume of 500 µL. An isocratic flow of 5 mL/min was used, with a mobile phase composed of acetonitrile–water (30:70) (v/v) with 0.1% of TFA. The oven temperature was 25°C

Test	Amount per injection	Recovery (%)	Purity (%)
1	2.6 ± 0.1 µg of 13-desMeC	76.2 ± 1.5	97.5 ± 1.3
	13.2 ± 0.4 µg of 13,19-didesMeC	86.4 ± 0.9	99.2 ± 1.1
2	8.2 ± 0.2 µg of 13-desMeC	70.2 ± 1.4	92.0 ± 0.8
	54.8 ± 0.9 µg of 13,19-didesMeC	81.4 ± 1.6	98.0 ± 1.7
3	8.2 ± 0.2 µg of 13-desMeC	60.4 ± 1.2	97.1 ± 1.2
	54.8 ± 0.9 µg of 13,19-didesMeC	63.4 ± 1.3	98.2 ± 0.5
4	3.36 ± 0.6 µg of 13-desMeC	79.5 ± 1.2	97.6 ± 1.5
	28 ± 0.9 µg of 13,19-didesMeC	80.6 ± 1.3	98.7 ± 2.0

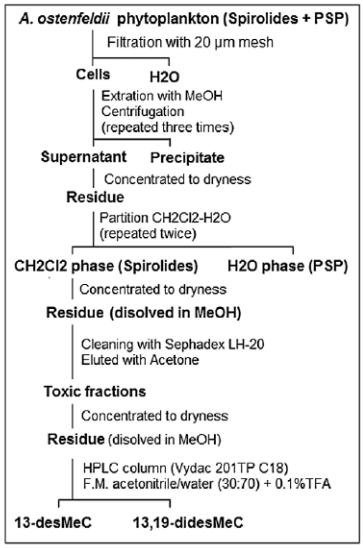


Figure 6. Scheme for the extraction and chromatographic purification of 13-desMeC and 13,19-didesMeC toxins.

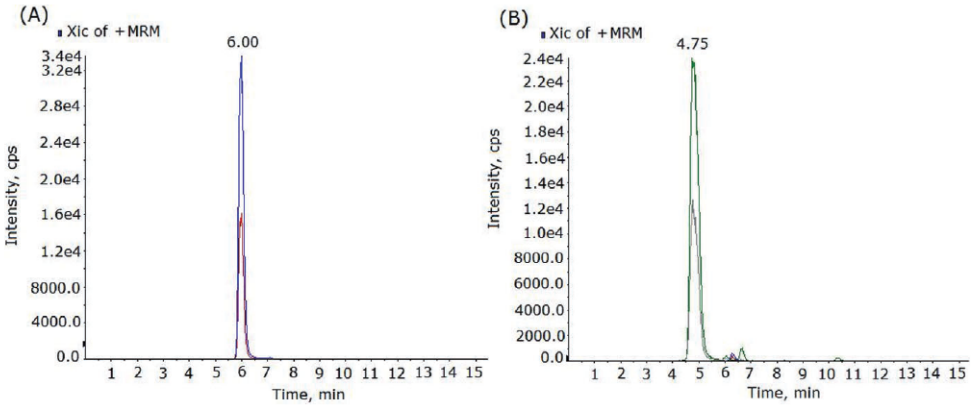


Figure 5. Chromatogram corresponding to LC-MS analysis in MRM positive mode of 13-desMeC (A) and 13,19-didesMeC (B) purified by preparative HPLC system. The purities achieved were 99.9 ± 0.5% for 13-desMeC and 98.7 ± 2.0% for 13,19-didesMeC.

hexane to eliminate lipophilic interferences. The method described here omitted the partition against hexane because the extraction was done from phytoplankton biomass extracts with little or no lipid fraction. 13-desMeC was isolated by others from cells without performing any partition, only with organic solvent extractions and cleaning in a Sep-Pak C-18 plus cartridge (Ciminiello *et al.*, 2006). Spirolides A, C and 13-desMeC were isolated from extracts of contaminated scallop digestive glands by performing methanol extractions, dissolving in 70% MeOH–30% H_2O and partitioning against C_6H_{12} (Hu *et al.*, 2001). Equally, spirolides were identified in Norwegian shellfish by conducting partitions with chloroform after aqueous methanol extractions (Aasen *et al.*, 2005). In order to achieve the complete extraction, we carried out three partitions water: dichloromethane (1:1) to get the spirolides in the organic fraction.

In this paper, data show that dichloromethane is an efficient solvent for removing spirolides from water after performing extraction with methanol. Moreover, it is also an essential step for the isolation of these toxins from *A. ostenfeldii* strains that also produce paralytic toxins. The PSP are hydrophilic compounds and have affinity for water. Hence, this partition separated the two groups of toxins that *A. ostenfeldii* dinoflagellate produce.

The sample cleaning was conducted with Sephadex LH-20. The literature recommends adding the toxins in dichloromethane and using methanol as a solvent elution for these toxins. Using these solvents, we obtained very low recoveries, so other organic solvents were tested. Ethanol presents a polarity index of 5.2, and methanol 5.1, which was not enough to elute the toxins. Acetonitrile–water is the mobile phase utilized in LC-MS and spirolides elute with 50:50 solvent proportions, but poor results were obtained when this solvent was used. Finally, the sample was dissolved in methanol because spirolides were stable in this solvent and eluted with acetone. Also, good recovery data were obtained when the elution was done with acetonitrile–water (50:50) and the sample was loaded in methanol, but fewer pigments were retained in the matrix gel when the sample was eluted with this solution instead of acetone.

The last step in the isolation of 13-desMeC and 13,19-didesMeC was preparative LC with fractions collection based on their *m/z* values. The mass-triggered mode was considered more efficient due to its higher sensitivity and better selectivity. The collection in UV-triggered mode was not used because in this method undesirable compounds are collected if they absorb in the same wavelength. Moreover, the mass-triggered mode is advisable in sample combinations to ensure a high purity in the collected fractions and achieve better signs in chromatograms. After the collection, the fractions were dried and dissolved in methanol with 0.05% TFA. This compound gives acidity to the medium.

The performance of a chromatographic separation process can be evaluated by several parameters representing various aspects of the efficient purification method, such as the purity and recovery. High purities have been obtained in this method: 99% for 13,19-didesMeC and 96% for 13-desMeC with recoveries of 82.8 ± 1.6% for both toxins. Comparing several chromatograms of injections performed on different days, both spirolides eluted in the same order with similar retention times. An important concern in method quantification is the availability of calibration standards. Marine toxins are expensive and difficult to acquire in high purity. Therefore, the method described herein shows for the first time a complete and efficient protocol to separate and purify spirolide toxins produced by *A. ostenfeldii* dinoflagellate.

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I.3. Primer ensayo directo de polarización de la fluorescencia para la detección y cuantificación de SPXs en muestras de mejillones.

Resumen

En 2009, nuestro grupo de investigación propuso el primer ensayo de polarización de la fluorescencia (FP) para detectar CIs. En esta publicación se diseña un nuevo ensayo de FP para la cuantificación directa de SPXs. Este nuevo método, resultó en una mejora de sensibilidad, rapidez y sencillez. En el diseño del método, se utilizaron nAChR de membranas del pez *Torpedo marmorata* marcados con un derivado de la fluoresceína. Los dos SPXs utlizados para el ensayo, 13-desMeC y 13,19-didesMeC se extrajeron y purificaron de cultivos de *A. ostenfeldii*. Los resultados muestran una disminución en la FP al aumentar la concentración de las toxinas. De este modo, se obtiene una relación entre las unidades de FP y la cantidad de SPXS en una muestra. Este ensayo directo es un método reproducible, simple y muy sensible con un LOD sobre 25 nM para 13-desMeC y 150 nM para 13,19-didesMeC. Este procedimiento se utilizó para medir SPXs en muestras de mejillones utilizando un protocolo de extracción y limpieza compatible con el ensayo de FP. Los resultados obtenidos muestran que este método es adecuado para cuantificar 13-desMeC en el rango de 50-350 µg/ Kg de carne. Otras toxinas liposolubles no interfieren con el ensayo, mostrando que es un método específico. Además, la presencia de matriz no afecta en el rango de concentraciones de toxina que están relacionadas con el un riesgo de intoxicación por SPXs.

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First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples

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ABSTRACT

In 2009, we achieve the first inhibition FP assay to detect imine cyclic toxins. In the present paper we propose a new FP assay for direct quantify spirolides. This new method has resulted in significant improvement of sensitivity, rapidity and accessibility. In the method design, nicotinic acetylcholine receptor from *Torpedo marmorata* membranes labelled with a derivative of fluorescein was used. Spirolides, 13-desmethyl spirolide C (13-desMeC) and 13,19-didesmethyl spirolide C (13,19-didesMeC) were extracted and purified from cultures of the *Alexandrium ostenfeldii* dinoflagellate. Data showed the decrease of FP when toxin concentration was increased. Thus, a relationship between the FP units and the spirolides amount present in a sample was obtained. This direct assay is a reproducible, simple and very sensitive method with a detection limit about 25 nM for 13-desMeC and 150 nM for 13,19-didesMeC. The procedure was used to measure spirolides in mussel samples using an extraction and clean up protocol suitable for the FP assay. Results obtained show that this method is able to quantify 13-desMeC in the range of 50–350 $\mu g\,kg^{-1}$ meat. Other liposoluble toxins did not interfere with the assay, proving a specific method. Moreover, the matrix do not affect in the range of toxin concentrations that involving risk of spirolides intoxication.

1. Introduction

Spirolides are a kind of macrocyclic polyether neurotoxins produced by the planktonic marine dinoflagellate *Alexandrium ostenfeldii* (*A. ostenfeldii*). These toxins are members of the cyclic imine group that includes other compounds such as gymnodimine, pteriatoxins, pinnatoxins, prorocentrolide, spiro-prorocentrimine and symbioimines [1–3]. Spirolides were first identified in extracts of the digestive glands of mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*) from the Atlantic coast of Nova Scotia, Canada, in 1991 [4]. Eventually, that year, a routine toxin monitoring of bivalve molluscs in this place revealed a toxic response in mice after intraperitoneal injections of shellfish extracts.

The occurrence of pteriatoxins, pinnatoxins, prorocentrolide, spiro-prorocentrimine and symbioimines is restricted to Japanese, Taiwanese and Chinese strains. Indeed, Pinnatoxin A has been linked to several major shellfish poisoning events in Japan and China [5]. The presence of Gymnodimine was confirmed in few locations such as New Zealand, Tunisia and Canada [4]. However, spirolides have been detected in a great number of species and locations around the world. Apart from Canada, these toxins have been reported in USA (Gulf of Maine) [6], Spain [7], France [8], Italy [9], Scotland [10], Norway [11] and Denmark [1] and Chile [12]. Therefore, spirolides have a global distribution range and they may be a risk to human health around the world. The mechanism of bioactivity of spirolides is not fully known. Preliminary pharmacological research into the mode of action of spirolides suggests that they are antagonists of the muscarinic acetylcholine receptor [4] and L-type transmembrane calcium channel activators in mammalian systems [13].

Chemically, spirolides are macrocyclic structures that contain spiro-linked tricyclic ether groups in addition to the heptacyclic imine ring. So far, seven main compounds designated with the letters A–G and several derivatives belonging to the C, D and G spirolide class have been isolated and structurally characterized employing NMR techniques [3,14–16]. They were called 13-desmethyl spirolide C (13-desMeC), 13,19-didesmethyl spirolide C (13,19-didesMeC), 27-hydroxy-13,19-didesmethyl spirolide C (27-OH-13,19-didesMeC), 13-desmethyl spirolide D (13-desMeD) and finally, 20-methylG (20-MeG). All of them comprise molecular

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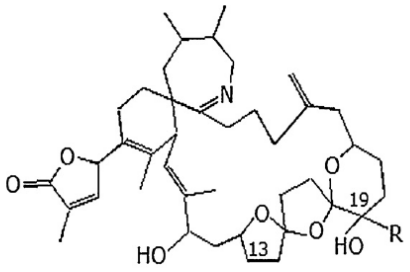

Spirolide	R	MW
13-desMeC	CH_3	691.5
13,19-didesMeC	H	677.5

Fig. 1. Chemical structures of 13-desmethyl spirolide C (13-desMeC) and 13,19-didesmethyl spirolide C (13,19-didesMeC).

weight from 677.5 to 711.5. Apart from some substituent radicals, the main differences found from spirolides class are the open imine ring from spirolide E and F and the unusual 5:6:6-trispiroketal ring system observed in spirolide G and its derivatives. This feature has not been found in other marine toxins [1] and its toxicity is not yet described. Nevertheless, the lack of the imine cyclic group in the spirolides E and F gives rise to a lack of toxicity in the mouse bioassay. In the rest of groups, small changes on the structure of spirolide compounds originate significant changes in the toxicity. Basically, spirolides from C group are more toxic than spirolides A, B and D, after intra peritoneal administration to mice.

New subclasses of spirolide marine toxins, represented by spirolides H and I, have recently been isolated from the dinoflagellate *A. ostenfeldii* [17]. These spirolides are structurally distinct from other spirolides in that they contain a 5:6 dispiroketal ring system rather than the trispiroketal ring system characteristic of previously isolated spirolides. Interestingly, spirolide H contains the cyclic imine moiety, but does not exhibit toxicity in the mouse bioassay, suggesting that the cyclic imine presence is not the only structural requirement for toxicity.

At present, legislated limits for spirolides toxins do not exist, but its great recurrence in numerous countries and the toxicological information obtained in the mouse bioassay indicate that these compounds constitute a group of toxins that should be worldwide regulated. Among the analytical technologies for spirolide detection is the liquid chromatography with mass detection. But the investigations in this field are restricted by the lack of pure compounds. To date, 13-desMeC is the only one available certificated standard. This shortage of pure compounds needed for calibration and adjustment of equipments makes difficult the validation of these technologies in the next years. Therefore, it is necessary to search alternative options to the chemical methods and mouse bioassay to control spirolide toxins.

In the present paper, it is described a new fluorescence polarization (FP) method that detects and quantifies 13-desMeC and 13,19-didesMeC (Fig. 1) in mussel extracts by the binding of the nicotinic acetylcholine receptor from *Torpedo marmorata* membranes. The assay is based on the change in fluorescence polarization of fluorescently labelled nicotinic receptor when bound by a spirolide toxin. This functional assay reporting the first demonstration of a direct FP technique dedicated to the spirolide detection.

2. Materials and methods

2.1. Reagents and materials

13-desMeC and 13,19-didesMeC were extracted and purified from cultures of *A. ostenfeldii* strain CCMP1773 following previous work [18]. Briefly, the cultures grew in seawater enriched with F/2 and Walne culture mediums at 28‰ of salinity. The dinoflagellates were shaken gently by hand and the growth occurred at 19 °C. Cells were extracted four times by adding methanol followed by sonication. After centrifugation, the methanolic supernatants were pooled, evaporated to dryness, dissolved in water and partitioned three times with dichloromethane. The organic layer was dried, dissolved in acetone and loaded to a Sephadex LH-20 column and the toxins were eluted with methanol. The fractions were evaporated and dissolved in methanol. Chromatographic separation of spirolides was performed by a LC preparative and analytical system coupled to a mass spectrometer from Waters, using 250 mm × 10 mm i.d. column packed with 5 μm Vydac 201TP (C18) and 5 mL min^{-1} of phase mobile composed of acetonitrile/water (30:70, v/v) with 0.1% (v/v) trifluoroacetic acid (TFA).

Pure azaspiracid-1 and okadaic acid were purchased from Laboratorios Cifga (Lugo, Spain). Phosphate-buffered saline solution (PBS) employed in this study was made with the followings compounds: 137 mM NaCl (Panreac), 8.2 mM Na_2HPO_4 (Panreac), 1.5 mM KH_2PO_4 from Merk (Darmstadt, Germany), 3.2 mM KCl (Panreac), pH adjusted to 7.3 adding NaOH (Panreac). Dichloromethane and methanol were purchased from Panreac (Barcelona, Spain).

Black 96-well polystyrene microplates, Microtiter Microfluor® 1 (from Thermo scientific, Hudson, NH, USA), flat-bottom, were used in all experiments.

2.2. Nicotinic acetylcoline receptor

T. marmorata fish were obtained alive from the Station Biologique de Roscoff (France), and kept in artificial seawater for about a week in the aquarium of the CNRS animal house in Gif sur Yvette, until been used to prepare membranes from the electric tissue. *Torpedo* electrocyte membranes rich in $\alpha 1_2\beta\gamma\delta$ nicotinic acetylcholine receptors (nAChRs) were purified in a cold room (4 °C) according to procedures previously described [19] with some modifications, as reported recently [20]. Membranes enriched in nAChR were resuspended in 5 mM glycine and stored at −80 °C.

The receptor was used in PBS (pH 7) composed of 130 mM NaCl, 1.5 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, 0,1% Tween-20 (v/v) and 0.1% BSA (w/v).

2.3. Fluorescence polarization

A derivative of fluorescein, succinimidyl esther of carboxyfluorescein (FAM), was employed as fluorescent molecule. Receptor labelling was performed using a kit purchased from emp Biotech: Fluoro protein 498 Spin Labelling and Purification Kit (Berlin, Germany). This article includes all chemicals, tools, and also the dye reactive, needed for the labelling and purification processes. The receptor is mixed with a sodium bicarbonate solution, the dye is added and the mixture reacts for 1 h at room temperature and protected from light. In order to avoid unspecific interactions or unstable ester bond formation between dye and protein, this kit provides hydroxylamine for use as a stop reagent. Finally, spin columns are used for rapid and efficient purification of the receptor–dye conjugate. With this kit, the dye reacts with an amine group of the protein and forms a covalent amide linkage. The protein–dye conjugates have fluorescence-excitation and

fluorescence-emission maxima at around 498 nm and 522 nm, respectively.

The fluorescence polarization was measured by means of the Multi-Mode Microplate Reader, Synergy™ 4 from Biotek (Winooski, Vermont, USA). The instrument detection modes include fluorescence Intensity, fluorescence Polarization, time-resolved fluorescence, luminescence, and UV–visible absorbance. Two types of fluorescence detection systems are available with Synergy 4, filter-based and monochromator-based. The instrument is equipped with dichroic mirrors and polarizing filters for fluorescence polarization. For measurement of FP and Fluorescence, excitation and emission wavelengths of 485/20 and 528/20 were used for protein–dye conjugates, respectively.

The polarization degree of the emitted light (measured in millipolarization units, mP) is calculated by the following equation:

$$\mathrm{mP} = 1000\frac{I_V - GI_H}{I_V + GI_H}$$

where I_V is the fluorescence intensity measured with vertical polarization excitation filters and vertical polarization emission filters (named parallel intensity), I_H is the fluorescence intensity measured with vertical polarization excitation filters and horizontal polarization emission filters (named perpendicular intensity), and G is a correction factor that accounts for the optical components of the instrument that affect the light beam depending on its polarization plane.

The instrument was controlled using BioTekïs Gen5™ PC software for all measures, and data were exported to excel software for analysis. All results in this study are expressed as the mean ± SEM and the experiments were performed in triplicate with duplicate measurements per replicate.

2.4. LC–MS detection

In order to know the toxin content and percentage of recovery in each step, aliquots from extraction method were analyzed by means of LC–MS. The equipment used was a combination of HPLC plus mass detector. The HPLC system, from Shimadzu (Kyoto, Japan), consisted of two pumps (LC-10ADvp), autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp) and system controller (SCL-10Avp). This system was coupled to a QTRAP LC/MS/MS system from Applied Biosystems (USA), which consisted of a hybrid quadrupole-linear ion trap mass spectrometer equipped with an API fitted with an ESI source. Nitrogen generator NM20ZA was from Peak Scientific (Billerica, MA, USA).

The column used for spirolide separations was a 2 mm × 50 mm BDS-Hypersil-C8 analytical column with a particle size of 3 μm and a 10 mm × 2.1 mm guard cartridge from Thermo (Waltham, MA, USA). The temperature was set at 25 °C. The mobile phase consisted of two components: water (A) and acetonitrile/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 30–90% B for 8 min, then, 90% B and 10% A were hold for 3 min and reducing afterwards to 30% B over 0.5 min. Then, they were hold again for 2.5 min until the next run. The mobile phase flow rate was 0.2 mL min^{-1} and the injection volume was 5 μL. Collision-induced dissociation (CID) in the ion-trap MS was carried out on the protonated molecule $[M+H]^+$, for each toxin. The ESI source of QTRAP was operated with the following optimized values of source-dependent parameters: Curtain gas™: 15 psi, collision-activated dissociation gas (CAD): 6 psi, IonSpray Voltage: 4000 V, temperature: 450 °C, gas 1: 50 psi and gas 2: 50 psi. The Multiple Reaction Monitoring (MRM) experiments were performed by selecting the following groups of transitions: 692.5 > 674.5 and 692.5 > 444.5 (for 13-desMeC) and 678.5 > 660.5 and 678.5 > 430.5 (for 13,19-didesMeC).

2.5. Processing of mussels samples

An amount of 1.5 kg of mussels was purchased from market, opened and the meat was homogenized with a blender. The extract was divided in 10 g aliquots, and stored at −20 °C until use. Then, to evaluate the matrix effects on the FP assay, the extracts of mussel were thawed and three extraction methods were employed. In Method 1 (M1), 10 g of mussel homogenate was extracted with 30 mL of acetone and then, extracted with the same volume of methanol. After pooling the supernatants, they were evaporated in a MiVac centrifugal concentrator from Genevac (Ipswich, UK). The residue was dissolved in 400 μL methanol: PBS (3:1) and passed through 0.45 μm filters (Millipore Ultrafree-MC centrifugal filter units, Bedford, MA, USA). In Method 2 (M2), 10 g of mussel homogenate was twice extracted with 30 mL of methanol and the supernatants were pooled and concentrated. The matrix was dissolved in 400 μL of methanol: PBS (3:1) and filtrated by the same way than M1. In Method 3 (M3), 30 mL of methanol was added to 10 g of mussel homogenate. This extract was centrifuged at 3000 rpm for 10 min at 4 °C, and the residue was extracted twice with 30 mL methanol. The supernatants were combined and concentrated. The pellet was then, dissolved in 60 mL of water and partitioned twice against 60 mL of CH_2Cl_2. The organic layer was evaporated, dissolved in 1 mL of methanol: PBS (4:6) and passed through 0.45 μm filters.

3. Results

Although the mechanism of action of spirolides in cells has not been fully clarified, the experimental evidence suggests that muscarinic and nicotinic receptors are involved in their mode of action [21–23]. Therefore, we designed a direct detection method for spirolides based on nicotinic acetylcholine receptor (nAChR) target using FP. In this method, one of two compounds should be fluorescent, for this reason, a derivate of fluorescein was used and the membrane-receptor was labelled. In order to select the proper dilution factor and volume of dye-membrane-receptor in each experiment, the intensity of the reaction of labelling was measured by dissolving an aliquot of nAChR-F conjugate in different volumes of PBS buffer. Finally, a dilution factor of 1:2500 was employed, well final volume 250 μL. The membrane-receptor-F conjugate was divided into 5 μL aliquots and stored at −80° C until use. Seven molecules of dye per molecule of membrane-receptor were used. Therefore, the concentration of dye-membrane-receptor in PBS solution remains constant in all experiments.

First, the binding of 13-desMeC to membranes enriched in nAChRs was tested. Different concentrations of 13-desMeC ranging from 10-1000 nM were added to the same amount of membrane-receptor-F conjugate and then, FP was measured after 10 min. First, the incubation was performed at room temperature, however, the binding was not observed (data not shown). Then, the incubation was done at 37 °C. As shown in Fig. 2A, a decrease of FP units was observed when the toxin concentration was increased. The signal decreased from 298 mP units for 10 nM to 172.75 mP units for 1000 nM. The data can be fitted as a Gaussian curve. To evaluate the binding validity, two parameters should be considered, the fluorescence intensity of conjugate in the presence of toxin and the interference of solvent on FP. Fig. 2B shows the measures of the fluorescence intensity of membrane-receptor-F conjugate when different concentrations of 13-desMeC were added. As it is shown, the information provided by FP, Fig. 2A, is lost when total fluorescence intensity is the parameter to consider, as shown in

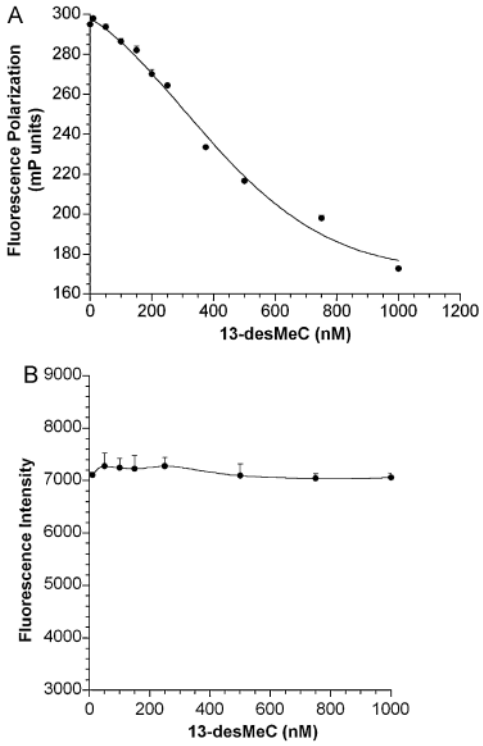

Fig. 2. Variation of (A) FP units and (B) Fluorescence intensity of membrane-enriched with nAChRs-F conjugate in the presence of 13-desmethyl spirolide C toxin (10–1000 nM) measured after 10 min incubation. Each point of the curve represents the mean ± SEM of $n = 4$ assays. The mP units were obtained from the microplate reader expressed as raw data.

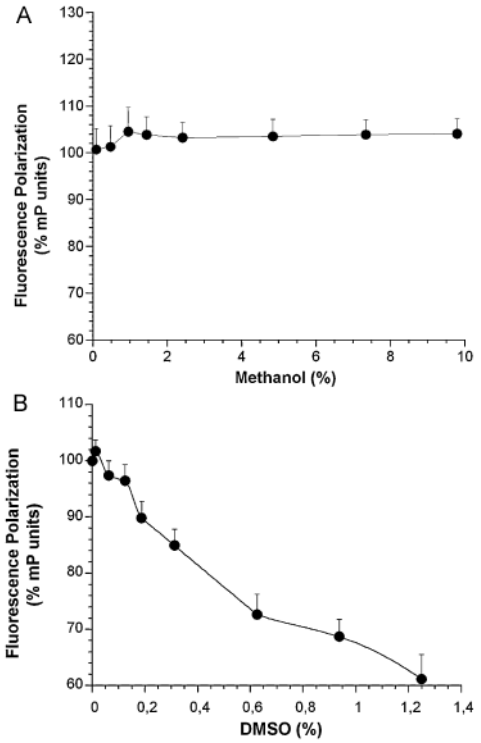

Fig. 3. Effect of (A) methanol and (B) DMSO on FP units of membrane-enriched with nAChRs-F conjugate. Different amounts of solvent were added to a constant amount of labelled membrane-enriched nAChR. FP was measured after 10 min incubation. Abscissa axis represents the final solvent proportion obtained in the well. Ordinate axis represents the FP as percentage response in relation to control response. Data are means ± SEM of 3 different experiments.

Fig. 2B. This spectroscopic characteristic remains in a constant average value, with slight fluctuations that cannot be related to the concentration of toxin. The toxins were diluted in methanol plus PBS buffer. Therefore, the interference of solvent was evaluated by doing serial dilutions of methanol in PBS until reach the same percentage of solvent found in those solutions with toxin. These results are shown in Fig. 3A. These values are expressed as percentage of response in relation to control response, that is, the FP signal of membrane-receptor-F conjugate without methanol. The highest content of solvent was 9.8% of methanol. No fall in the signal was observed at this methanol concentration. Besides methanol, the use of another solvent as DMSO was tested in this FP assay. This organic compound is usually employed to dissolve lipophilic marine toxins. The experiment was done using the same amount of labelled membrane-receptor and solutions of DMSO in concentrations from 0.0125% to 1.25% without any toxin. In this case, as Fig. 3B shows, the FP is also expressed as percentage (%) and falls from 100% to 61% in the presence of 1.25% of DMSO in the well. Therefore, this solvent interferes in this method even at concentrations as low as 0.2%.

The next step was to study the effect of incubation time on the spirolide-membrane-receptor binding. Three incubation times and toxin concentrations ranging from 10 to 250 nM were checked. The toxin was added to the same quantity of membrane-receptor-F conjugate and then, three incubation times 10, 30 and 60 min at 37 °C were done. Fig. 4 collects results obtained in this assay.

The mP units versus toxin concentration were plotted and the calibration curve fitted linear either after 10 or 30 min incubation, Fig. 4A and B. However, for longer incubations (60 min, Fig. 4C), the values are closer to a curve. From these results, the best fit is achieved after 10 min.

After selecting temperature, incubation time and solvent, different toxin concentrations ranging from 10 to 500 nM were checked. As shown in Fig. 5 a linear relationship was obtained. The FP response is expressed in percentage, setting 100% for the control value, which is the membrane-receptor-dye without toxin, and 0% for the maximum amount of toxin employed that corresponds to 500 nM. FP signal gave a straight line with a correlation coefficient of 0.99245 in this concentration range. Thus, a direct relationship between FP units and toxin concentration was obtained, and this relationship allowed determining the 13-desMeC concentration in a sample if the FP units are measured. The limit of detection (LOD) and limit of quantification (LOQ) calculated in these conditions were 25 nM and 75 nM, respectively.

The specificity of the FP method for detect and quantify spirolides was checked using two lipophilic toxins, azaspiracid-1

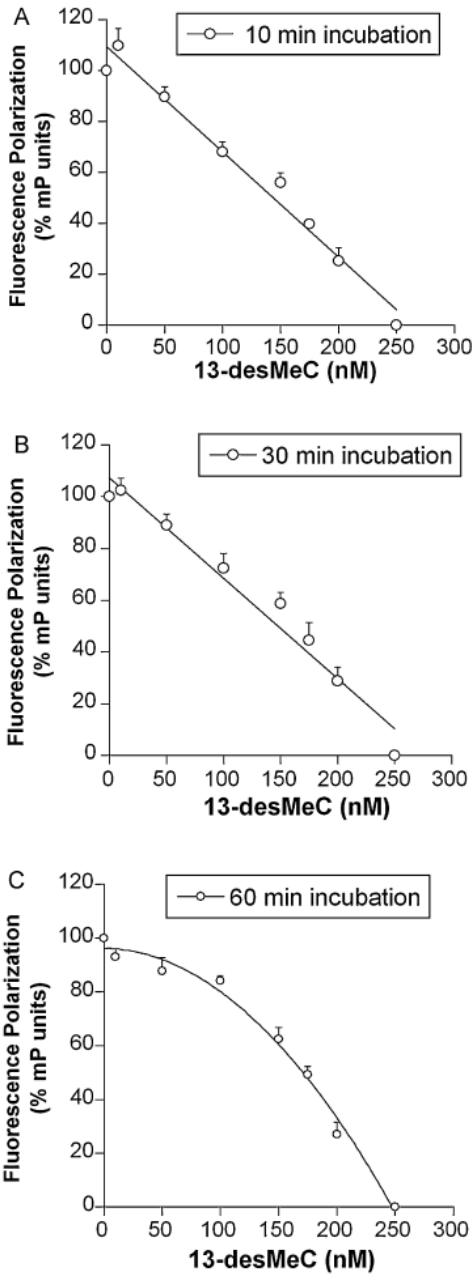

Fig. 4. Effect of 13-desMeC on FP units of membrane-enriched with nAChRs-F conjugate obtained for different incubation times. Variation of FP units (% mP) as a function of different toxin concentration in PBS medium. The control value corresponds to the fluorescence intensity of membrane-enriched with nAChRs-F conjugate in PBS medium without any toxin. Data are means ± SEM of 8 experiments. Toxin concentration are ranging from 10 to 250 nM. FP from (A) was measured after 10 min incubation, (B) after 30 min and (C) after 60 min. Data from (A) and (B) were fitted by linear regression. Data from (C) by polynomial curve.

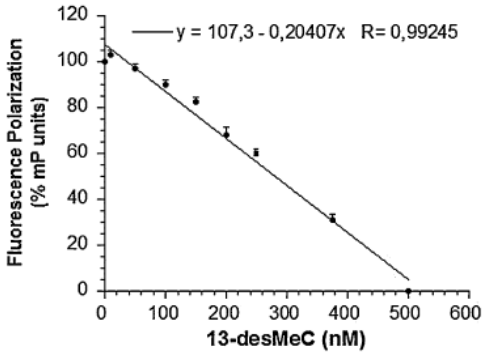

Fig. 5. Change in FP units (%) of membrane-enriched with nAChRs-F conjugate. Toxin concentrates are ranging from 10 to 500 nM and they are plotted as a straight line, setting as 100% the FP value obtained for control and 0% the FP value achieved for 500 nM. Each point of the curve represents the mean ± SEM of $n = 4$ assays.

and okadaic acid that usually coexist with spirolide toxins in contaminated shellfish. The effect of both toxins on the method was tested in the same experimental conditions than spirolides. The results are shown in Fig. 6. Standards of azaspiracid-1 and okadaic acid were dried and dissolved in the same proportion of methanol plus PBS. Then, different concentrations of both toxins were added to the same amount of membrane-receptor-F conjugate and FP was measured after 10, 30 and 60 min of incubation. The results evidence that azaspiracid-1 and okadaic acid does not bind to nAChR and, therefore, they do not interfere with the FP assay at final concentrations as high as 250 nM and 500 nM, respectively.

Subsequently, it was tested the FP method for another spirolide analogue, the 13,19-didesMeC. First, the binding of membrane-receptor-F conjugate and 13,19-didesMeC was checked in a concentration range 0 to 250 nM. These concentrations did not affect to the FP. For this reason, the concentration was increased to 1200 nM. Fig. 7 shows FP values after three incubation times 10, 30 and 60 min. FP values were adjusted to a straight line after 10 and 30 min incubation, Fig. 7A and B. However, after 60 min incubation (Fig. 7C), the values are closer to a curve. From these results, the best fit is achieved after 10 min. After selecting the incubation time, different 13,19-didesMeC concentrations ranging from 10 to 1000 nM were checked, Fig. 8. Thus, a direct relationship between the FP units and different concentrations of 13,19-didesMeC was obtained, correlation coefficient of $R = 0.98245$. From this graphic, LOD, 150 nM 13,19-didesMeC, and LOQ, 200 nM 13,19-didesMeC, were calculated. In summary, this FP method is also accurate to quantify 13,19-didesMeC.

In order to evaluate the assay in a food matrix, where the spirolides may be a risk for human health, samples of mussel spiked with 13-desMeC were employed. The aim was to provide an extraction method of spirolides from mussels, as simple and easy as possible with high percentage of toxin recovery and without interferences of matrix in detection method. First the three extraction methods described in Section 2, M1, M2 and M3 were used with blank mussel. Briefly, M1 includes one extraction with acetone and another one with methanol, M2 includes two extractions with methanol and M3 three extractions with methanol followed by a partition with CH_2Cl_2. In these conditions FP val-

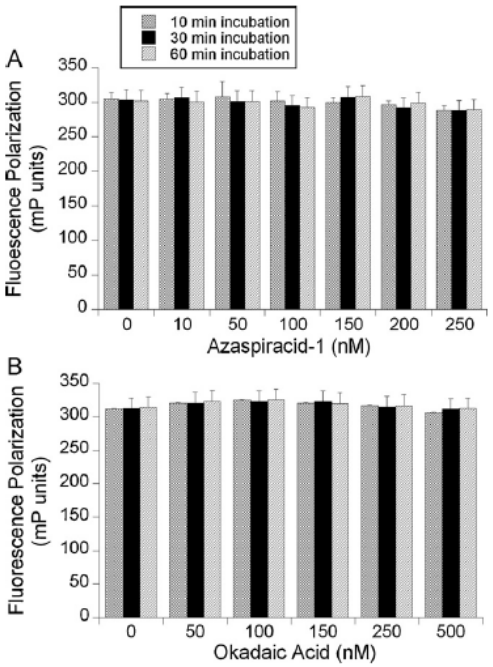

Fig. 6. FP units of membrane-enriched with nAChRs-F conjugate in the presence of different concentrations of azaspiracid-1 (A) and okadaic acid (B). The effect of both lipophilic toxins on FP was tested in the same conditions used in the spirolide assays. FP was measured after 10, 30 and 60 min incubation. Each stick represents the mean ± SEM of $n = 3$ experiments.

ues obtained for control solution, membrane-receptor-F conjugate in PBS, were compared with the FP values of membrane-receptor-F conjugate in PBS in the presence of 25 μL of mussel extract after these extraction protocols. The results are shown in the Fig. 9. The raw values obtained for M1 and M2 were 139 ± 11 mP and 169 ± 10 mP, respectively, while the results achieved for the control were 314.09 ± 3.12 mP. Therefore, from these results, other clean up steps for the matrix should be needed. However, the FP raw value obtained after M3 was 313.89 ± 8.92 mP and the control 314.09 ± 3.12 mP. Therefore, this extraction protocol is the most suitable to detect spirolide toxins in mussel samples using the FP technique.

Then, mussel samples were spiked with 1.25 μg 13-desMeC toxin and the protocol M3 was used to extract the toxin from mussel matrix. In each extraction step, one aliquot was saved to evaluate the toxin recovery percentage by means of LC–MS and to compare the final results obtained for both FP and LC–MS methods. In brief, 10 g of contaminated mussel homogenate were extracted three times with 30 mL methanol. The supernatants were combined after centrifugation and concentrated. An aliquot was analyzed by LC–MS resulting in 1.271 ± 0.100 μg 13-desMeC. The pellet was dissolved in 60 mL water and partitioned twice against 60 mL of CH_2Cl_2. 2 organic layers were pooled and one aliquot was measured. The LC–MS result was 1.219 ± 0.028 μg 13-desMeC. Therefore, there was no toxin loss in the extraction process. The CH_2Cl_2 layer was evaporated, dissolved in 1 mL methanol: PBS (4:6) and passed through 0.45 μm filters. This final extract was analyzed by LC–MS and the FP method and the results obtained

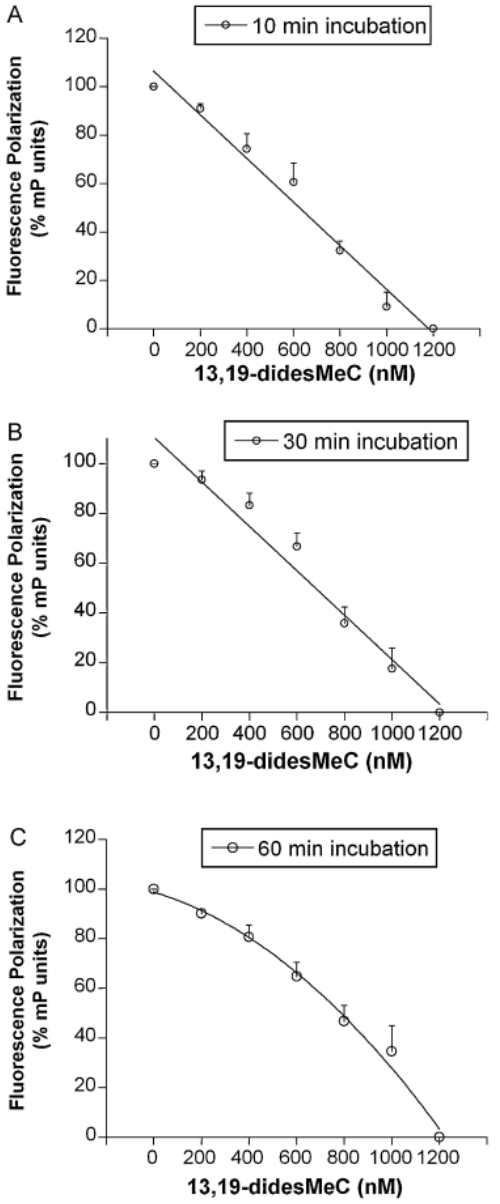

Fig. 7. Effects of 13,19-didesMeC toxin on FP of membrane-enriched with nAChRs-F conjugate achieved for three incubation times. The descent on FP units (mP) is according to different toxin concentration in PBS medium. The control value corresponds to the fluorescence intensity of membrane-labelled nAChR in PBS medium without any toxin. FP was measured after 10 min incubation (A), after 30 min (B) and after 60 min incubation (C). Data from (A) and (B) are plotted as a linear regression. The values from (C) form a Gaussian curve. Data are means ± SEM of 8 experiments.

Table 1
Toxin amount employed to spike the mussels and toxin amount detected by FP direct method and LC-MS method after the extraction procedure. Each value represents the mean ± SEM of $n = 3$ experiments.

13-desMeC toxin amount in mussels	Determination by FP assay	Determination by LC–MS
1.250 μg	1.164 μg ± 0.049	1.100 μg ± 0.03

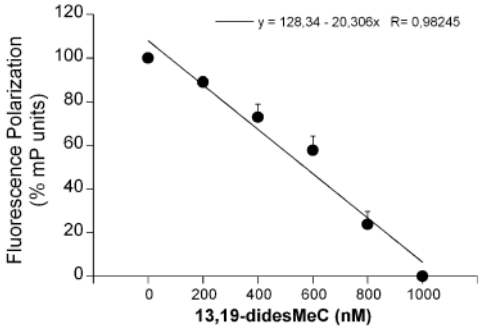

Fig. 8. Variations in FP units (%) of membrane-enriched with nAChRs-F conjugate induced by different 13, 19-didesmethyl spirolide C (13,19-didesMeC) concentrations. Data obtained for 200, 400, 600, 800, 1000 nM of 13,19-didesMeC toxin are plotted as a straight line representing as 100% the FP value obtained for control, that is, the FP value obtained for membrane-labelled nAChR without any toxin, and 0%, the FP value achieved for 1000 nM.

are shown in Table 1. The 13-desMeC toxin amount detected by FP assay in the mussel extract was 1.164 μg ± 0.049 while it was 1.100 μg ± 0.03 by LC–MS. Thus, the percentage of toxin recovery was 88.00% ± 0.02. In addition, comparable results were obtained for both techniques.

Table 2 collects the advantages of the present method compared to the previous Vilariño 2009 and shows the most representative features of both approaches. This new design has significantly improved the FP assay in terms of sensibility, rapidity, accessibility. In summary, the present method is more sensitive, faster, easier and simpler than the previous and constituted an efficient direct assay

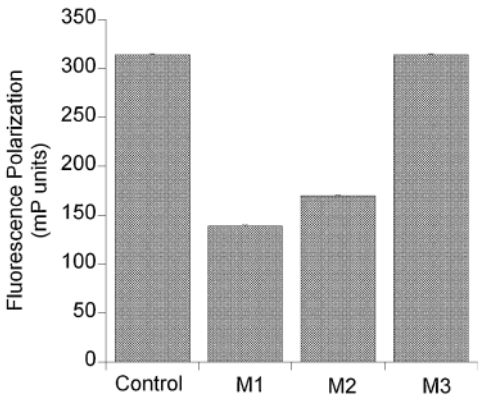

Fig. 9. Effect of the different matrixes mixed with membrane-enriched with nAChRs-F conjugate on mP units, after cleaning them up by three methods: Method 1 (M1), Method 2 (M2) and Method 3 (M3). Control represents FP values of membrane-enriched with nAChRs-F conjugate on PBS. The measures were performed after 10 min incubation. Each stick represents the mean ± SEM of $n = 3$ experiments.

to detect and quantify 13-desMeC in the range of 50–350 μg kg^{-1} shellfish meat.

4. Discussion

The frequency of occurrence and intensity of harmful algal blooms appear to be increasing on a global scale. Monitoring of toxins in seafood and risk assessment for human exposure is the main objective of food control. Consumption of seafood contaminated with marine toxins may cause serious diseases [24–27]. Therefore, the analysis of shellfish for marine toxins is an important labour that must be conducted according to international regulations and according to the restrictions set. Although spirolide toxins are not still regulated, their toxicological data and their presence in many countries have led to set toxic limits in toxicology meeting. In general, the propose amount for cyclic imine toxins is 0.4 mg kg^{-1} meat [28].

So far, the mouse bioassay has been the most important international method for the detection of algal toxins, and all analytical methods designed to the validation as official methods have to be evaluated against bioassays. However, biological tests are not completely satisfactory, due to the low sensitivity and the absence of specificity. Moreover, there is growing resistance against the use of animal experiments. For these reasons, many efforts have been made to determine algal toxins with alternative methods to bioassay [29–33]. Although the mechanism of action of spirolides in cells is not yet well understood, experimental evidence suggest that spirolides might act through nAChRs [21,23,34,35].

Our interest was focused on the design of detection methods for imine cyclic toxins based on their pharmacologic target and to propose functional assays that could replace the mouse bioassay method. Vilariño et al. 2009 describes the first method to detect gymnodimine A and 13-desMeC published by our group [20]. Later on, the matrix effect of several mollusc species was analyzed, in a quantification range for 13-desMeC of 85–700 μg kg^{-1} of shellfish meat [36]. Subsequent, this method was checked with 13,19-didesMeC [37]. The basis of all these previous assays is that the presence of the cyclic imines in solution inhibited the interaction of fluorescent-labelled alpha-bungarotoxin with membrane-enriched nAChRs in a concentration-dependent manner [20]. However, it is well know that the competitive assays present numerous disadvantages over the non-competitive ones. They require the use of extra analogues, are typically less sensitive and slow, and provide a shortest linear range [38].

In view of these considerations, the need to search for new alternative detection methods to the mouse bioassay, led to further research in this field and to propose a direct assay, based on binding of the toxin to receptors, without using fluorescent alpha-bungarotoxin.

The method described in this paper does not follow the standard FP approach. FP methods are typically performed using small fluorescent molecules which rotate quickly and yield low FP values. When a larger molecule binds to them, a large complex forms and will rotate slower and yield higher FP values. Nevertheless, bibliography describes several direct assays focused on the detection of the small molecules. In 1981, a direct assay to detect the binding between avidin (MW ~ 66–69 kDa) and biotin (MW ~ 244 Da) was published by using fluorescein-labelled avidin

Table 2
Summary of the advantages of the present method compared to the previous Vilariño, 2009.

Advantages (present method)	Vilariño, 2009 method	Present method
1. Innovative design	Inhibition assay	1st direct assay to detect spirolides by FP
2. Greater sensitivity	85 μg 13-desMeC kg	50 μg 13-desMeC kg
3. Larger linear range (easier quantification)	100–250 nM	10–500 nM
4. Greater accessibility	It requires alpha-bungarotoxin labelled with Alexa Fluor 488 dye	It does not require the synthesis of extra fluorescent analogues
5. Sorter incubation (faster)	2 h + 30 min	10 min
6. Simpler extraction (fewer steps)	Extraction acetone (3) Partition water: hexane (1) Partition chloroform (3)	Extraction methanol (3) Partition water: dichloromethane (1)

[39]. Following this design a FP method to detect yessotoxin (MW ~ 1142 Da) by using fluorescein-labelled phosphodiesterases (MW ~ 120 kDa) was developed [30,40]. A similar design was performed to analyze digoxin (MW ~ 780 Da) by using a uniform-labeledscFvimmunoreagent [38]. The decrease in FP units observed when the big molecule is labelled can be due to structural changes after bound the other molecule. Thus, 2 types of motions define the movement of the dye: global and local. Global motion is the movement that decreases when the molecular volume increases. This relation often corresponds with an increase in the molecular weight, however, this does not always happen. For example, the fluorescence polarization of a dye bound to a single strand of DNA is bigger than the fluorescence polarization of a dye bound to a DNA duplex with the same molecular weight [41], maybe due to the different volumes of the two structures. Other important parameter is the local motion of the dye. For example, the FP of a tryptophan residue of a protein generally increases when it binds to another macromolecule. However, this polarization can decrease if the local motion of the tryptophan increases appreciably on binding. This situation occurs when the elongation factor Tu (EF-Tu) binds to the elongation factor Ts (EF-Ts). In fact the FP of the complex EF-Tu-GDP, which has a molecular weight of 43,000 is greater than the FP of the complex EF-Tu-EF-Ts which has a molecular weight of 74,000 [42]. The same happen when the polarization of a primer is higher than the polarization of the PCR product despite the fact that the large size of the latter [43]. All these references can be applied in our study, so we suggest that the binding between spirolides and the labelled nAChRs produces an increase in the local motion of the dye and so, a decrease in its FP.

For the development of the method, several parameters such as incubation time, solvent type and toxins concentration were evaluated and optimized. Three incubation times were checked, 10, 30 and 60 min. Data show that 10 min is a sufficient and efficient incubation time for binding spirolides to the membrane-receptor, discarding the other two times. Thus, a more rapid detection method is obtained. Methanol, even though at final concentration in the well of 9.8%, is a suitable solvent to dissolve the spirolides in this FP assay. It was verified that changes in FP were induced by the interaction of spirolide to membrane-nAChRs and the solvent did not interfere with experiments. However, DMSO in high concentrations affects mP units of nAChR–F conjugate, therefore this solvent could not be used as toxin solvent within this assay.

The quantifying ranges for both toxins were assessed. First, the experiments were performed with 13-desMeC toxin. It was observed that between 75 and 500 nM it could establish a direct relation with a good linear correlation index of $R = 0.992$. The slope and R values are high, showing that this equation is appropriate to quantify spirolides. Then, the detection method was successfully applied for 13,19-didesMeC, but in this case, this analogue causes smaller changes in FP than 13-desMeC. This suggests that nAChRs might have lower or different affinity for 13,19-didesMeC than 13-desMeC. Finally, the linear range was assessed to be 200–1000 nM for 13,19-didesMeC.

To evaluate the selectivity of this method, the cross-reactivity with other lipophilic marine toxins such as azaspiracid-1 and okadaic acid were investigated. These two toxins are frequently found in marine plankton with spirolide toxins [44–46]. The results obtained demonstrated that azaspiracid-1 and okadaic acid did not interfered with the FP method, resulting in a specific method.

The matrix effect was also tested. Spirolides are lipophilic compounds which occur naturally in amounts of 103 μg kg^{-1} mussel meat [11]. Therefore, the method was tested in 10 g of mussel homogenate spiked with 1.25 μg of 13-desMeC. 13-desMeC was selected because it causes a large decreased of FP using small amount of toxin. The matrix effects may exist in FP technique and therefore, an extensive study was performed for the method application to mussel samples. It was necessary to check the matrix interference on FP units. After preliminary experiments, it was possible to design an adequate clean up procedure with high toxin recovery. Thus, the method here propose is able to quantify 13-desMeC toxins in the range 50–350 μg kg^{-1} meat while the previous method [20] has a limit of quantification of 75 μg kg^{-1} meat. Greater sensitivity and greater recovery for 13-desMeC is achieved in this matrix extraction procedure with respect to previously FP method published [36]. A simpler extraction protocol using fewer steps is provided with a percentage of toxin recovery of 88%. In addition, comparable results were obtained by LC–MS and FP. This fact confirms the reliability of this functional assay and point to FP as an easy, fast and simple alternative of LC–MS method to monitor spirolides.

In summary, this assay constitutes a sensitive method to detect spirolides in samples with a low limit of detection of 25 nM for 13-desMeC and 150 nM for 13,19-didesMeC. Thus, the functional assay described in this paper is a solid choice to measure spirolides concentrations in various samples, including contaminated mussels matrix for human consumption.

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I.4. Farmacocinética y toxicología de SPXs administrados por vía oral e i.p.

Resumen

Este estudio muestra por primera vez una descripción detallada y completa sobre la sintomatología observada en un ratón cuando se administran SPXs por vía i.p. También se compara la toxicidad i.p del 13-desMeC, 13,19-didesMeC y 20-MeG en experimentos realizados con toxinas de alto grado de pureza. El bioensayo indica que 13-desMeC y 13,19-didesMeC son compuestos extremadamente tóxicos con una LD_{50} de 27,9 µg/ kg and 32,2 µg/ kg, respectivamente. Sin embargo, cuando 20-MeG se administra i.p. con dosis de hasta 63,5 µg/ kg, no se registraron muertes. Con el objetivo de evaluar la toxicidad oral, se administraron SPXs a ratones mediante intubación gástrica. Posteriormente, se recogieron muestras de sangre, orina y heces y se analizaron por LC-MS/MS. Los SPXs aparecen en la sangre a los 15 min y en la orina después de una hora de ser administrados oralmente. En resumen, en esta publicación, se proporcionan nuevos datos sobre la toxicidad, absorción y excreción de los SPXs en ratones. Esta información es importante para la regulación en la UE de los SPXs.

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Pharmacokinetic and toxicological data of spirolides after oral and intraperitoneal administration

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ABSTRACT

Spirolides are a kind of marine toxins included in the cyclic imine toxin group and produced by the dinoflagellate *Alexandrium ostenfeldii*. This study shows for the first time a complete and detailed description about the symptoms observed in mice when these toxins were intraperitoneal (i.p.) administered. It is also compared the i.p. toxicity of 13-desmethyl spirolide C (13-desMeC), 13,19-didesMeC (13,19-didesMeC) and 20-methyl spirolide G (20-Me-G) in experiments performed with highly purified toxins. The bioassay indicates that 13-desMeC and 13,19-didesMeC are extremely toxic compounds which have a LD_{50} of 27.9 μg/kg and 32.2 μg/kg, respectively. However, when 20-MeG was i.p administrated with dose up 63.5 μg/kg, no deaths were recorded.

In order to evaluate the oral toxicity, spirolides were administered by gastric intubation into mice. Then, samples of blood, urine and faeces were collected and analyzed by liquid chromatography–mass spectrometry tandem (LC–MS/MS) technique. Spirolides appear in blood at 15 min and in urine after 1 h of being toxin administered. In summary, in this paper, it is provided new data about the toxicity, absorption, and excretion of spirolides in mouse. So far, little information is available on this item but necessary for spirolide regulation in the European Union (EU).

1. Introduction

Spirolides are lipophilic compounds classified in the cyclic imine toxin group. This group includes other lipophilic compounds such as gymnodimine, pinnatoxins, prorocentrolide, pteriatoxins, espiro-prorocentrimine and symbioimines (MacKinnon et al., 2004, 2006a,b). Spirolide toxicity was first detected in a routine biotoxin monitoring of bivalve molluscs for DSP toxins in Nova Scotia (Canada) in 1991 (Hu et al., 1996; MacKinnon et al., 2004). An unusual rapid mouse death after intraperitoneal (i.p.) injection of lipophilic extracts of scallops and mussels revealed a highly potent toxic response in mice (MacKinnon et al., 2006b). The symptoms observed were different from those associated with known shellfish toxins, including those responsible for DSP or PSP intoxication (MacKinnon et al., 2006b).

Nowadays, spirolides have a global distribution range. In addition to Canada, these toxins have been reported in USA (Gulf of Maine) (Anderson et al., 2005), Scotland (John et al., 2003), Norway (Aasen et al., 2005), Spain (Villar Gonzalez et al., 2006), France (Amzil et al., 2007), Italy (Ciminiello et al., 2006), Denmark (MacKinnon et al., 2006a) and Chile (Álvarez et al., 2010). Although spirolides were detected in many countries around the world, they were not linked to human intoxications. Other than spirolides, the other cyclic imines toxins have been recorded in few locations. The existence of pteriatoxins, pinnatoxins, prorocentrolide, spiro-prorocentrimine and symbioimines is restricted to Japanese, Taiwanese and Chinese strains (Munday, 2008). Pinnatoxin A was implicated in the most important shellfish poisoning events in Japan and China (Meilert and Brimble, 2006). The presence of Gymnodimine was also confirmed in few locations such as New Zealand, Tunisia and Canada (Meilert and Brimble, 2006) and, like spirolides, gymnodimines were not linked to human poisoning.

To date, spirolide production has been confirmed in phytoplankton species of the genus *Alexandrium* (*A. ostenfelii*/*A. peruvianum*).(Touzet et al., 2008). The mechanism of action of spirolides is not fully elucidated. Cholinergic (muscarinic and nicotinic) receptors have been proposed as the main target of these toxins. Preliminary pharmacological research suggests that spirolides are irreversible antagonists of the muscarinic acetylcholine receptor (Meilert and Brimble, 2006) and L-type transmembrane calcium

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channel activators in mammalian systems (Sleno et al., 2004). They were also identified as irreversible inhibitors of muscarinic receptors (Wandscheer et al., 2010).

So far, 14 compounds of spirolide marine toxins have been isolated and characterized from dinoflagellates cultures and mollusk extracts. Chemically, spirolides are polyether compounds that contain spiro-linked tricyclic ethers. Some of them have distinctive features that allow their classification in various subclasses. The main class comprising the spirolides A, B, C, D, some des-methyl derivatives of spirolides C and D, namely 13-desmethyl spirolide C (13-desMeC), 13,19-didesmethyl spirolide C (13,19-didesMeC) and 13-desmethyl spirolide D (13-desMeD) and the hydroxylated analogue 27-hydroxy-13,19-didesmethyl spirolide C (27-OH-13,19-didesMeC). These toxins have a 6:5:5-polyether ring system in addition to the heptacyclic imine ring. Spirolides E and F contain the same polyether ring system, but not cyclic moiety. Spirolide G and their variant 20-methyl spirolide G (20-MeG) have the imine ring intact and contain an unusual 5:5:6-trispiroketal ring system, never observed before in other marine toxins group (MacKinnon et al., 2006a). Finally, a group of spirolide represented by spirolide H and I was characterized (Roach et al., 2009). These toxins are structurally distinct from the other spirolides previously isolated. They contain a 5:6 dispiroketal ring system rather than the trispiroketal ring system. Until this discovery, the lack of toxicity observed in the mouse bioassay following i.p. injections of extracts containing spirolide E and F was associated with the absence of imine cyclic group. Interestingly, spirolide H contains the cyclic imine moiety, but does not exhibit toxicity in the mouse bioassay, suggesting that the cyclic imine presence is not the only structural requirement for toxicity (Roach et al., 2009).

Although there have been no reports of toxic effects in humans due to ingestion of spirolides, its great recurrence in numerous countries and the symptoms observed in the mouse bioassay indicate that these compounds constitute a group of toxins that should be regulated worldwide. Lack of toxicological data prevents the establishment of legislated limits for spirolides and in general for the cyclic imines toxins group (Panel, 2010). No information is presently available about the metabolism of spirolide in animals. In this study, efforts are made to further improve the knowledge about oral and i.p. toxicity of spirolides and their toxicological effects.

2. Materials and methods

2.1. Reagents and mice

Tween® Monostearate (Tween 60) was purchased from Sigma–Aldrich (Germany). Physiologic saline solution for toxin administration was from Laboratorios Grifols, S.A. (Barcelona, Spain). Nutritive solution for oral administration into mice, Sueroral Casen, was purchased from Laboratorios Casen-Fleet (Utebo, Zaragoza, Spain). The composition per litre of water was: NaCl (3.5 g), kCl (1.5 g), trisodium citrate dehydrate (2.9 g) and glucose (20 g). Methanol and acetonitrile, were from Panreac Quimica S.A. (Barcelona, Spain). Formic acid and ammonium formate were purchased from Merck (Darmstadt, Germany).

Pure 13-desMeC, 13,19-didesMeC and 20-MeG toxins were purchased from Laboratios CIFGA S.A. (Lugo, Spain). The ampoules contained 0.5 mL of solution with 10.2 μM 13-desMeC, 14.8 μM 13,19-didesMeC and 16.9 μM 20-MeG in methanol with 0.05% (v/v) TFA.

Swiss Mice, weighing 19–21 g were obtained from the animal care facilities of the University of Santiago de Compostela (USC) and were housed and manipulated according to the guidelines of the USC Bioethical Committee. All possible efforts were made to reduce animal suffering and minimize the number of animals used.

2.2. Intraperitoneal toxicity

In order to know the i.p. toxicity, toxins were administrated dissolved in physiological saline solution with 1% Tween® Monostearate (Tween 60). Stock solutions of 13-desMeC, 13,19-didesMeC and 20-MeG were done in methanol with 0.05% TFA and the toxin dilutions for physiological studies were made from these solutions. Controls animals received in the saline solution the same percentage of methanol and TFA.

2.3. Oral toxicity

Before performing the studies to evaluate the oral toxicity, mice were deprived of food for 24 h and then, they had free access to nutrient solution during the experiment. The toxins administrated orally were dissolved in physiological saline solution with 1% Tween® Monostearate (Tween 60) and the percentage of methanol administrated was lower than 0.8% always. These solutions were administered by gastric intubation directly into the oesophagus and after toxins administration mice were fed *ad libitum*. In order to evaluate the toxicity, blood (from 450 to 700 μL), urine (70 μL) and faeces (from 0.77 to 2.26 g) were analyzed. Controls animals received in the saline solution the same percentage of methanol and their blood, urine and faeces were also checked. Blood and urine were collected after 15 min, 1 h and 24 h of toxin administration and the faeces were collected for 24 h. Spirolides were detected in the biological samples by means of LC–MS/MS (Liquid Chromatography–mass spectrometry tandem) after toxin extraction. For this, an equal volume of methanol than the blood was immediately added to the blood and the mixture was centrifuged at 13000 rpm, 10 min. Subsequently, the supernatants were dry and dissolved in 100 μL methanol. To ensure complete toxin solubilisation, each sample was sonication at least 1 min. Finally, the extracts were filtered though 0.45 μm filters to be analyzed by LC–MS/MS. Urine samples were dried, dissolved in 50 μL methanol and filtered through 0.45 μm filters. Faeces samples were homogenized, extracted with methanol and centrifuged at 3000 rpm for 5 min. Then, the pellet was twice extracted with the same solvent. Subsequently, organic extracts were pooled, dried and dissolved in 2 mL methanol. Finally the extract was filtered though 0.45 μm filters and analyzed by means of LC–MS/MS.

Tissues analyzed were heart, kidneys, liver, spleen, stomach and gastrointestinal tracts. They were removed from mice after 24 h and 7 days of being toxin administrated. Control and treated animals were euthanized with CO_2 after 24 h or 7 days of treatment. At necropsy, the skull was opened and the brain was removed and fixed by immersion in buffered 10% formalin and Bouin. Considerable caution was taken when handling the brain to avoid the induction of artefacts. The remaining tissues collected were placed in separate containers with the same fixative. All tissues were processed for paraffin embedding and sections (5 μm) were mounted on subbed slides. Control and treated animals were handled under the same conditions and tissue samples were processed at the same time. Sections from internal organs were stained with Mayer's haematoxylin and eosin (H&E) for routine histological evaluations. Coronal sections of brain were also stained with H&E.

2.4. LC–MS/MS analysis

The equipment used is a combination of HPLC plus mass detector. The HPLC system, from Shimadzu (Kyoto, Japan), consists of two pumps (LC-10ADvp), autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp) and system controller (SCL-10Avp). This system is coupled to a QTRAP MS/MS system from Applied Biosystems, (USA), which consists of a hybrid quadrupole-linear ion trap mass spectrometer equipped with an API fitted with an ESI source. The nitrogen generator is a Nitrocraft NCLC/MS from Air Liquide (Spain).

The column used for spirolide separations was a 2 × 50 mm BDS-Hypersil-C8 analytical column with a particle size of 3 μm and a 10 × 2.1 mm guard cartridge from Thermo (Waltham, MA, USA). The temperature was set at 25 °C. The mobile phase consisted of two components: water (A) and acetonitrile/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 30–90% B for 8 min, then, 90% B was hold for 3 min and reducing afterwards to 30% B over 0.5 min and hold for 5.5 min until the next run. The mobile phase flow rate was 0.2 mL/min and the injection volume was 5 μL. Collision-induced dissociation (CID) in the ion-trap MS was carried out on the protonated molecule, $[M+H]^+$, for each toxin. The ESI source of QTRAP was operated with the following optimized values of source-dependent parameters: Curtain gas™: 15 psi; collision-activated dissociation gas (CAD): 6 psi; IonSpray Voltage: 4000 V; temperature: 450 °C; gas 1: 50 psi and gas 2: 50 psi. The Multiple Reaction Monitoring (MRM) experiments were performed by selecting the following groups of transitions: 692.4 > 674.4 and 692.4 > 444.4 (for 13-desMeC) and 678.4 > 660.4 and 678.4 > 430.4 (for 13,19-didesMeC).

3. Results

First, in order to known the symptomatology caused by spirolides, mice were i.p. injected with several doses of 13-desMeC, 13,19-didesMeC (from 0.14 to 1 μg/mL) and 20-MeG (from 0.5 to 1.3 μg/mL). The rapid onset of symptoms showed that spirolides were highly toxic when they are i.p. injected. The behaviour was clearly neurological and symptoms were seen just after injection, indicating a rapid adsorption of the spirolide toxins from the peritoneum. The mice were hyperactive immediately after dosing, but in a few minutes, the movement became slower with several shakes appearing. When 13-desMeC and 13,19-didesMeC were

administrated in doses above 0.5 μg/mL, mice legs became paralysed and extended. These mice subsequently became completely immobile with pronounced respiratory distress and marked abdominal breathing. The mice showed urine and dregs and then, the respiratory rate became progressively slower, until it ceased. Pronounced convulsions were observed shortly before death. At toxic, but sub-lethal dose levels, respiratory distress was recorded, but the mice recovered within 30 min to an apparently normal state, and no adverse effects were observed during the subsequent 24 h observation period. When 20-MeG was i.p. administrated, only the high dose, more than 1 μg/mL, induced paralysis in legs and immobilization with respiratory distress. However, after being 10 min immobilized, mice recovery and no deaths were recorded for any dose, even in the higher concentration checked, 1.3 μg/mL.

In order to know the time of death and the time when symptoms appeared, a new assay was done. In this experiment the animal behaviour from toxin injection to mouse death was registered. The aim of this study was to know the time when first symptoms appeared, time when the mouse is immobilized and the time from immobilization to death. For this, a dose of 1 μg/mL of 13-desMeC, 13,19-didesMeC and 20-MeG were i.p. administered. First symptoms were considered as the onset of slower movement and uncoordination. As Fig. 1 shows, symptoms appeared in 1.8 ± 0.2 min for 13-desMeC, however, 6.4 ± 3.5 min and 7.1 ± 0.5 for 13,19-didesMeC and 20-MeG were necessary for the occurrence of the first symptoms. The time from toxin injection to mice immobilization, was also lower for 13-desMeC, resulting in 3.8 ± 0.7 min, 9.7 ± 4.6 min for 13,19-didesMeC and 12.5 ± 1.5 min for 20-MeG. Mice remained paralyzed for a long time when they were injected with 20-MeG and no deaths were observed. However, all mice administered with 13-desMeC and 13,19-didesMeC died. Death time was 8.2 ± 1.1 min for 13-desMeC and 12.0 ± 2.1 min for 13.19-didesMeC. Next step was to determinate the LD_{50} values only for the two more toxic compounds, since no deaths were recorded for 20-MeG. For this, 13-desMeC and 13,19-didesMeC were administrated by i.p. injection into dose of 0.14, 0.50, 0.60 and 0.80 μg/mL for each toxin. For the experiment design, it was considered to inject as minimum four mice for dose and to register the number of deaths. Results are shown in Fig. 2. All mice injected with 0.14 μg/mL remained alive and without symptoms after being controlled several hours. The results for 0.50 and 0.60 μg/mL revealed that 13-desMeC LD_{50} was an intermediate value between these two doses, since for 0.50 ng/mL, 1 out of 5 mice died and, for the dose of 0.60 ng/mL, 3 out of 4 mice died. However, when 0.50 ng/mL 13,19-didesMeC were i.p. injected, only one mice died and for 0.60 ng/mL, 2 out of 5 mice died. All animals injected with 0.80 μg/mL died within a few minutes. Then 0.55 μg/mL 13-desMeC and 0.62 μg/mL 13,19-didesMeC were injected. As Fig. 2 shows, 0.55 μg/mL 13-desMeC killed 25% of the population and 0.62 μg/mL 13,19-desMeC were lethal to 75%. Finally, 0.57 μg/mL 13-desMeC and 0.61 μg/mL 13,19-didesMeC were i.p. injected and 50% of mice died. Therefore, considering the mice weight, LD_{50} are 27.9 μg/kg for 13-desMeC and 32.2 μg/kg for 13,19-didesMeC.

In order to know the spirolides oral toxicity, only the two more toxic toxins were checked. Thus, toxins were administered to mice per-os through a cannula with a single dose of 27.9 μg/Kg 13-desMeC or 32.2 μg/Kg 13,19-desMeC or methanol carrier for control. These animals were food deprived for 24 h before the experiment and feeding only with nutritive solution with salts and glucose. These animals were sacrificed at 15 min, 1 h, 24 h and 7 days of toxin administration. Samples of blood, urine and faeces were collected at 15 min, 1 h and 24 h and analyzed by LC–MS/MS for the toxin presence. The identification and quantification of spirolide toxins in the biological samples were performed against 13-desMeC and 13,19-didesMeC standards. Fig. 3 shows the chromatograms of a urine sample collected after spirolide toxins

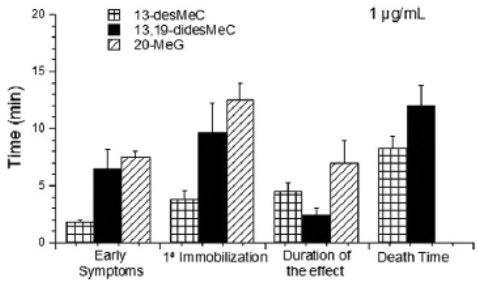

Fig. 1. Registration of different stages and some symptoms observed from administration to mouse death after i.p. administration of spirolides at dose of 1 μg/mL for each toxin. Each stick represents the mean ± SEM of seven mouse injections.

administration. Fig. 3a corresponds to urine sample after 13-desMeC per-os administration and Fig. 3b, urine sample collected after 13,19-didesMeC per-os administration. The quantity of 13-desMeC and 13,19-didesMeC detected in all samples are shown in Table 1 and the amounts are expressed in ng/mL for blood and urine samples and ng/g for faeces samples. The limit of quantification (LOQ) for both spirolides was 1 ng/mL in methanol. As it can be observed in this table, both spirolides were detected in blood at 15 min, the quantity was bigger for 13-desMeC than 13,19-didesMeC. After 1 h of being i.p. injected, high amounts of spirolides were detected in urine, once again more quantity of 13-desMeC than 13,19-didesMeC and trace amounts for both toxins in blood. After 24 h, big amount of spirolides in faeces were detected, more quantity of 13,19-didesMeC than 13-desMeC and trace amounts in blood and urine. All mice remained alive; some showed hyperactivity symptoms, but they recovered in a few minutes.

Finally, analysis of the tissues (heart, kidneys, liver, spleen, stomach, small intestine and large intestine) were done. For this, mice were sacrificed at 24 h and 7 days of being toxin administrated with 27.9 μg/Kg 13-desMeC or 32.2 μg/Kg 13,19-desMeC. Immediately, the organs were extracted from the animals to perform the histological evaluations by H&E. None of the tissues analyzed by light microscopy showed any observable alteration neither after 24 h nor after 7 days of the oral administration. Tissues did not show any difference compared to the control animals.

From these results, after i.p. injection, 13-desMeC is slightly more toxic than 13,19-didesMeC and both are considerably more toxic than 20-MeG. Spirolides are absorbed by per-os administration and the availability in blood is greater for 13-desMeC than 13,19-didesMeC. After 24 h and 7 days, there are no direct injuries in the organs and the spirolides are excreted through urine and, fundamentally, through faeces.

4. Discussion

At present, an European regulation for spirolides toxins does not exist, but the symptoms observed in mouse bioassay for some spirolides and the frequent occurrence of these toxins reported in recent years require the accumulation of information to determine an appropriate level for regulatory actions. This is even more important given the irreversible nature of the binding of spirolides to their receptors, both muscarinic (Wandscheer et al., 2010) and nicotinic (Bourne et al., 2010). The proposed threshold amount for cyclic imines acute reference dose is, in general, 0.4 mg/kg meat (Aune, 2008). Due to the neurotoxic symptoms of spirolides, some of the results shown in the present work were taken into account

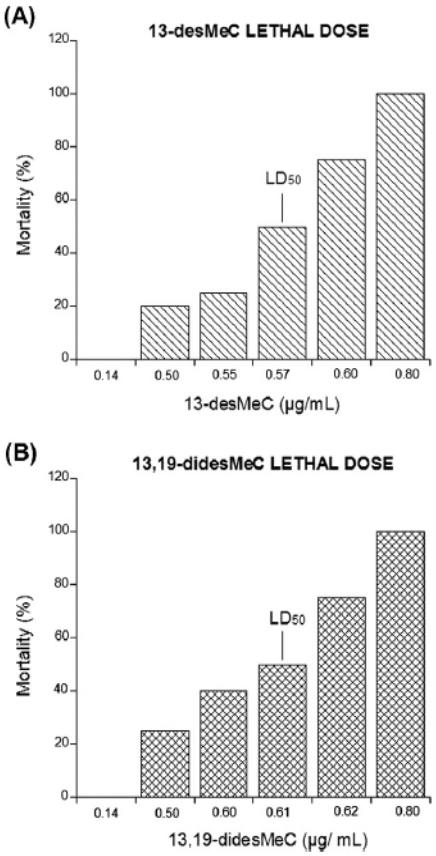

Fig. 2. Variations in the death percentage after i.p. injections of 13-desMeC (A) and 13,19-didesMeC (B) at different dose. Graphics includes the dose of 13-desMeC (A) and 13,19-didesMeC (B) required to kill half the members of a tested animal population (LD_{50}).

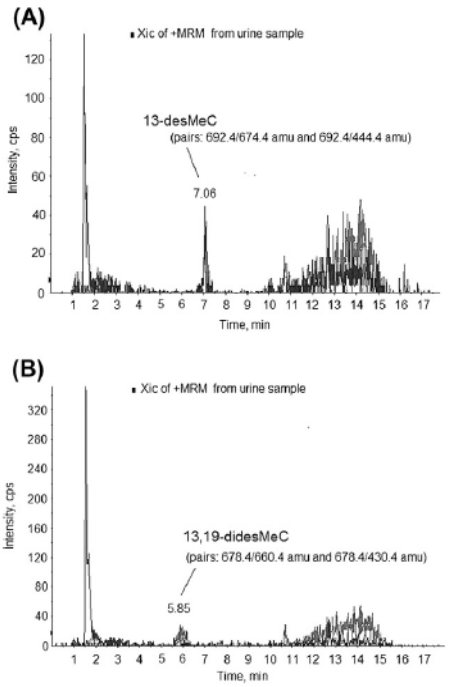

Fig. 3. Chromatograms of mice urine samples collected 1 h after 13-desMeC (A) and 13,19-didesMeC (B) oral administration.

by The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) to assess the risks to human health related to the consumption of spirolides (Panel, 2010).

Although muscarinic acetylcholine receptors are implicated in the mechanism of action of spirolide toxins (Gill et al., 2003; Aráoz et al., 2009; Bourne et al., 2010) there are still many unresolved questions associated with the intoxication syndrome in mammals. For example, in which extent these toxins are absorbed in the digestive systems, doubts about the toxin quantity absorbed, which is the concentration dynamic in plasma after several minutes of oral toxin administration and which is the following excretion route. These questions are addressed in this paper, which describes an experimental design that allowed to follow the kinetic of spirolide toxins poisoning *in vivo*, when an oral dose of 13-desMeC or 13,19-didesMeC was administrated to mice.

After per-os administration, toxins were found in the biological fluidics. If it is considered that the blood volume is 5% of body burden (1 mL for a 20 g mouse) (Bottein Dechraoui et al., 2007) and the toxin amount administrated was 560 ng 13-desMeC and 640 ng 13,19-didesMeC, our results revealed that toxin amount measured in mouse blood is small for both compounds. Despite this, the ratio is 1.6 times lower for 13,19-didesMeC than 13-desMeC. It suggested that 13-desMeC is absorbed in greater quantity than the other spirolide analogue. 1 h after the toxin is administrated, the amount detected per mL of urine is also higher for 13-desMeC than for 13,19-didesMeC since 1.41 and 1.01 ng 13-desMeC and 13,19-didesMeC were found, respectively. On the contrary, the toxin amount detected in faeces was slightly higher for 13,19-didesMeC than for 13-desMeC. Different quantities of both compounds in urine and faeces at the same time, suggest the evolution in the organism is different and therefore have different kinetic behaviour.

Recently, it was demonstrated by our group that 13-desMeC crosses a Caco-2 cells monolayer (Espiña et al., 2011). Trans-epithelial permeability assays using Caco-2 cells, a human adenocarcinoma cell line, is an excellent model to predict the intestinal absorption rate of different molecules obtaining better approximations to actual intestinal absorption rates when the compound tested is highly permeable. Using those tests we could predict that 13-desMeC would be absorbed in the human intestine. Therefore, this paper confirms those results.

Another import issue addressed in this study is the comparison of i.p. toxicity observed between the three spirolides. We obtained a LD_{50} of 27.9 µg/Kg for 13-desMeC, 32.2 µg/Kg for 13,19-didesMeC and no deaths were recorded for 20-MeG when doses up to 63.5 µg/kg were checked. This is the first report that set the LD_{50} for 13,19-didesMeC, shows the toxicity *in vivo* for this compound and compares the toxicity of 13-desMeC, 13,19-didesMeC and

Table 1
Quantity of 13-desMeC and 13,19-didesMeC detected in blood, urine and faeces. LOQ of 13-desMeC and 13,19-didesMeC was 1 ng/mL in methanol. Each value represents the mean ± SEM of $n = 3$ experiments.

	13-desMeC			13,19-didesMeC		
	Blood (ng/mL)	Urine (ng/mL)	Faeces (ng/g)	Blood (ng/mL)	Urine (ng/mL)	Faeces (ng/g)
15 min	0.28 ± 0.03			0.20 ± 0.05		
1 h	<LOQ	1.41 ± 0.03		<LOQ	1.01 ± 0.30	
24 h	<LOQ	<LOQ	4.07 ± 0.75	<LOQ	<LOQ	4.54 ± 0.47

20-MeG in a mouse bioassay done with highly pure compounds. So far, the LD_{50} of 20-MeG was set at 8 μg/kg (Munday, 2008). The only information about 13,19-didesMeC in the mouse bioassay is addressed by one toxicological study which affirms 13,19-didesMeC is five times less toxic than 13-desMeC by i.p. route. (MacKinnon et al., 2006b). In this assay, a minimum lethal dose of 13,19-didesMeC obtained by i.p. via in mice was 30 μg/kg. The authors compare this value with the LD_{50} 6.9 μg/kg obtained by i.p. injection of pure 13-desMeC performed by Munday (Munday, 2008). However, the LD_{50} value obtained by a mixture of spirolides that contained 13-desMeC was 40 μg/kg (Richard et al., 2001). The fact of obtaining different LD_{50} for the same compound can be due to the different toxin purity. For this reason, we perform this study with highly purified spirolides. Additionally, we observed that 20-MeG is less toxic than the others two spirolides. However, in a preliminary toxicology study (manuscript in preparation), after i.p. injections with 20-MeG into mice, this compound was detected in the brain. Analyses were performed by means of LC–MS/MS and the amount detected was near of 0.1% of the quantity injected.

The oral toxicity of spirolides seems to be significantly lower than its i.p. toxicity (Richard et al., 2001; Aasen et al., 2006). Spirolides were found to be orally more than 25 times less toxic to mice, compared with i.p. injections (Cembella and Krock, 2008). In fact, no human poisoning has been documented due to spirolide toxins. The reason may be that spirolides are quickly detoxified, since animals given sub-lethal doses were recovered only in few minutes, with no perceptible long-term effects. Spirolide appears to be rapidly eliminated in the urine. In any case, we cannot confirm if this lack of human poisonings is due to a higher rate of elimination of spirolides or to its transformation to a less potent molecule. Spirolides are stable in methanol or acidic aqueous methanol solution (Quilliam, 2003), therefore they may be stable under the acidic conditions of the stomach in the digestion process. Moreover, it was reported that the seven-member cyclic imine in spirolides C and D has been shown to be completely stable to hydrolysis upon treatment with aqueous acids (Hu et al., 2001; Pelc and Zakarian, 2005). Therefore, the spirolide molecule could pass intact into the blood. The presence of spirolides in urine after 24 h suggests that elimination of spirolide from the body takes less than 1 day. However, not all the toxin administrated was recovered. Spirolides could be metabolized and therefore, it would not be able to detect them by LC–MS/MS employing the mass spectrometer method (transitions) used for checking the parent spirolide molecule. Spirolides could also be distributed into different tissues despite the fact that no damage was observed. None of the tissues and organs analyzed by light microscopy showed any observable alteration neither 24 h nor 7 days of the oral administration. This fact can be due the assays was done with a non-lethal per-os dosage of spirolides.

In addition, spirolides could be absorbed and eliminated by urine which was not possible to recover due to urine samples were collected at punctual times. Spirolides were also detected in faeces. This fact suggests either that toxin are absorbed and eliminated or they are not absorbed and therefore, directly excreted.

The effects observed in mice for 13,19-didesMeC and 20-MeG were very similar those described for 13-desMeC toxin in previous studies (Gill et al., 2003). Regardless of the time when symptoms appeared, there is a clear difference between three toxins and these observations had not been described before. For a dose of 1 μg/kg, we observed the first symptoms at 1.8 min for 13-desMeC, 6.5 min for 13,19-didesMeC and 7.1 min for 20-MeG. The time up to immobilization follow the same order, 13-desMeC (3.83 min) < 13,19-didesMeC (6.68 min) < 20-MeG (12.50 min). However, surprisingly, mice i.p. injected with 13,19-didesMeC remain paralyzed less time than 13-desMeC despite the fact that it is less toxic. This could suggest both toxins bind to nicotinic acetylcholine receptor differently and therefore, have different toxic effect. The pharmacokinetic behaviour seems to be similar, however the death time is higher for 13,19-didesMeC than 13-desMeC. In fact, in a recent study, we observed that nicotinic acetylcholine receptors seems to have a lower affinity for 13,19-didesMeC than 13-desMeC (Otero et al., 2011) since the binding kinetic is not the same for both toxins after 10 min incubation.

In summary, this paper shows for the first time a complete description about the symptoms observed in mice for three spirolides and also compares the LD_{50} of 13-desMeC, 13,19-didesMeC and 20-MeG in assays performed in the same conditions, with highly purified toxins. The bioassay indicates that 13-desMeC is slightly more toxic than 13,19-didesMeC and both are considerably more toxic than 20-MeG. The study also shows spirolides are detected in blood, urine and faeces at different times. These observations suggest the toxic effect of a toxin which is currently not regulated. Data observed in the present study suggest that limits for spirolide toxins should be established.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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3.3. SECCIÓN II: CTXs. Estudio de la presencia de CTXs en las costas europeas.

La ciguatera es una intoxicación alimentaria humana causada por la ingestión de peces tropicales contaminados con las toxinas lipofílicas conocidas como CTXs. Estas toxinas son producidas por dinoflagelados de la especie *Gambierdiscus*. Los peces herbívoros acumulan estas toxinas en su musculatura y vísceras después de ingerir los dinoflagelados. Varios estudios epidemiológicos mostraron que la CFP es una enfermedad que se da en zonas con latitudes entre los 35º N y los 35º S, principalmente en áreas caribeñas, indo-pacíficas pero no en aguas cercanas al continente europeo y africano. Sin embargo, en los últimos años varias cepas de *Gambierdiscus* han aparecido en el mar Mediterráneo [113,115] y se ha descrito la presencia de CTXs en Canarias [114]. Estos hallazgos revelan una expansión de estas toxinas a nuevas zonas bien por la migración de peces o bien porque las especies del gº *Gambierdiscus* son capaces de adaptarse a aguas de estas regiones. De este modo, se esperan nuevos casos de CFP en el continente europeo. En esta sección se estudia la presencia de este grupo de toxinas en 2 especies de peces capturados en Europa de los que se sospecha una intoxicación por CTX.

A esta sección corresponde la publicación:

II. 1. First toxin profile of ciguateric fish in Madeira arquipelago (Europe)

II.1 Primer perfil de toxinas de peces con CTXs en el archipiélago de Madeira (Europa).

Resumen

En este estudio, se analizó una especie de *Seriola dumerilli* de peso 70 kg y una especie pequeña de *Seriola fasciata* capturadas en aguas pertenecientes a las islas Salvages (archipiélago de Madeira). Varios peces de este género habían sido implicados en casos sospechosos de ciguatera en este archipiélago portugués del océano Atlántico Norte. Los análisis se realizan por dos técnicas, un método

funcional utilizando células granulares de cerebelo y por cromatografía líquida de ultra eficacia acoplada a espectrometría de masas (UPLC-MS/MS). El estudio se llevó a cabo en una parte del músculo de la cola de *Seriola fasciata* y en cinco partes del cuerpo de *Seriola dumerili* (músculo de la cola, cabeza, músculos ventrales, músculo de la parte central y el hígado). El método funcional consiste en el estudio de la modificación de las corrientes de sodio en células granulares de cerebelo. Con el método químico se determina el perfil de toxinas de las muestras. Este método de UPLC-MS/MS, optimizado en este estudio, permite la separación y cuantificación de todos los análogos de CTXs presentes en las muestras. Después de una extracción y limpieza, los cromatogramas muestran la presencia de CTX-1B en 1111,6 *m/z*, CTX-3C en 1023,5 *m/z*, un análogo de CTX en 1040,6 *m/z*, y una CTX de la zona del Caribe o del Índico en 1141,6 *m/z*. Por lo tanto, los resultados obtenidos en este estudio por ambos métodos, confirman por primera vez, la presencia de CTXs en peces del archipiélago de Madeira.

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First Toxin Profile of Ciguateric Fish in Madeira Arquipelago (Europe)

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Ciguatera fish poisoning (CFP) is a human foodborne intoxication caused by ingestion of tropical fishes contaminated with the potent polyether toxins known as ciguatoxins (CTXs). These toxins are issued from *Gambierdiscus* species of dinoflagellates. Herbivorous fish accumulate these toxins in their musculature and viscera after ingesting dinoflagellates. Epidemiological studies showed that CFP has been present in areas between 35° North and 35° South latitude, mainly, Indo-pacific and Caribbean areas, but not in waters closed to European and African continent. In the present paper, a specimen of *Seriola dumerili* weighing 70 kg and a smaller *Seriola fasciata* specimen, captured in waters belonging to Selvagens Islands (Madeira Arquipelago), were analyzed. Fishes from this genus were implicated in previous suspected ciguatera poisoning outbreaks in the Portuguese Madeira Arquipelago in the North Atlantic Ocean. Analysis was performed by two approaches, a functional method using cerebellar granule cells and by ultraperformance liquid chromatography−mass spectrometry (UPLC-MS) method. The study was carried out in one portion of the tail muscle of *Seriola fasciata* and five parts of the body of *Seriola dumerili* (tail muscle, head, ventral muscle, mid muscle, and liver). The functional method consisted in the modification of the inward sodium current in cerebellar granule cells and the chemical method was a high resolution chromatography, which allowed elucidating the toxin profile in the samples. In addition, UPLC-MS technique was optimized and used for detecting and quantifying CTXs for the first time. After fish extraction and clean up, the chromatograms revealed the presence of CTX-1B at 1111.6 *m/z*, CTX-3C at 1023.5 *m/z*, a CTX analogue at 1040.6 *m/z*, and a CTX from the Caribbean or Indic waters at 1141.6 *m/z*. Therefore, the results obtained in the present paper for both methods confirm, for the first time, the presence of CTX in fish from Madeira Arquipelago.

The CFP is a common term used to describe the human intoxication due to ingestion of fish contaminated with CTXs. This disease is associated with the consumption of fish from several species of tropical and subtropical areas from Indian and Pacific Oceans and the Caribbean Sea. CTXs and CTX precursors are produced by *Gambierdiscus spp.* (Dinoflagellate). These microalgae are ingested by herbivorous fish and transferred though the trophic chain to the larger carnivores and human. These dinoflagellates are also capable of producing other types of potent marine toxins, such as maitotoxins, gambierol, and several gambieric acids.[1]

CTXs are considered a worldwide health problem. It has been estimated that approximately 20 000 cases of intoxication occur annually[2] and seems to be most prevalent in the South Pacific region.[3] Most blooming organisms require periodic monitoring in order to be correctly evaluated. Especially, *Gambierdiscus spp.* because it is not easily visible like other blooming organisms.[4] The symptoms of ciguatera begin with gastrointestinal problems, such as nausea, vomiting, diarrhea, and abdominal pain within 12 h of eating a toxic fish, and often abate within 24 h. Cardiovascular problems, such as bradycardia with hypotension may also be found during this acute period. From a few hours to 2 weeks after exposure, neurogical symptoms may appear. They comprise paresthesias, dysesthesias, and hyperesthesias.[5]

CTXs in finfish include an assemblage of principal CTX and numerous closely related structural isomers and congeners. Although the list of identified CTXs continues to grow,[4] nowadays there are approximately 20 Pacific CTXs and 2 Caribbean CTXs

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fully structured.[2,6,7] They have been isolated and characterized from tissues of different fish species including invertebrate feeders and benthic pelagic predators, with the peculiarity that herbivorous fish have never been involved in CFP in the Caribbean, in contrast to the Pacific.

The Pacific and Caribbean CTXs differ slightly in structure and toxicity. The major CTX involved in CFP in the Pacific region is CTX-1B. It typically contributes approximately 90% of the toxicity of ciguateric carnivorous fish.[8] CTX-1B structure elucidation was accomplished in 1989 with a molecular mass of 1110.6.[9] This molecule binds at site 5 of voltage-sensitive sodium channels, and it may cause lethality in mouse models with a LD_{50} of 0.25 μg/kg. The structure and configuration of the main CTX of the Caribbean area, C-CTX-1, was determined by nuclear magnetic resonance spectroscopy in 1998 with a mass of 1140.6.[10] This toxin was isolated from a horse-eye jack (*Caranx latus)*. Like CTX-1B, it is a potent sodium channel activator and exhibits a mouse LD_{50} of 3.7 μg/kg.

Chemically, the CTXs family is a group of cyclic polyether molecules soluble in polar solvents, highly oxygenated, with few double bonds and thermally stables to heat.[11] The main transformation of CTXs molecule occurs through the marine food chain, especially oxidative modifications which can enhance potency.[4] In fact, it seems CTX-1B results from the biotransformation of CTX-4B.[12]

In the present study we analyzed two species of fish, *Seriola dumerili* (Risso 1810) *(S. dumerili)* and *Seriola fasciata* (Bloch 1793) *(S. fasciata)*, caught at Selvagens Islands, part of Madeira Arquipelago (Portugal). Fishes belonging to *Seriola spp.* were implicated in previous cases of suspected CFP outbreaks.[13]

A cluster of mild neurological and gastrointestinal symptoms characteristic of CFP was first experienced among 6 vigilant keepers in charge of Madeira Natural Park at Selvagens Islands, starting in mid 2007 and lasting until mid 2008. These Islands are located 300 km south of Funchal (Madeira) and 160 km North of Santa Cruz de Tenerife (Canary Islands). In July 2008, a severe outbreak affected 11 crewmembers of a fishing boat.

In the first cases, the suspected fishes belonged to different species, but only those that had consumed *Seriola spp.* reported diarrhea. In the case of the crewmembers, severe symptoms onset at 4 h after consumption and included diarrhea, aching of muscles and joints, headaches, sensitivity in hands and feet, prostration, reversal of hot/cold temperature sensation, itchiness, numbness of the tongue and mouth, and numbness of the extremities. It was necessary to hospitalize them, and the recovery required more than a month in some cases. In this episode, the poisoning was attributed to a 30 kg *Seriola spp.* caught in Grande Selvagem Island. The July 2008 outbreak led to a clear diagnosis of ciguatera and helped in the understanding of the previous isolated outbreaks that were taking place with the vigilant keepers. Their incidents were prevented by avoiding fish consumption caught locally at Selvagens Islands.

The importance of this study was to found CTXs in these two fish species, being the first time that CTXs is described in these latitudes, (30° 04′ N, 15° 56′ W).

MATERIALS AND METHODS

Reagents and Toxin Standards. Methanol, dichloromethane, chloroform, hexane, sodium chloride, acetonitrile, periodic acid, and acetic acid were from Panreac Quimica S.A. (Barcelona, Spain). Formic acid and ammonium formate were purchased from Merck (Darmstadt, Germany). All solvents used in this study were high-performance liquid-chromatography or analytical grade, and the water was distilled and passed through a water purification system (Arium 611 Sartorius Gottingen, Germany).

A synthetic standard of CTX-3C was provided by Dr. Masahiro Hirama. The methodology applied for the synthesis was described for the first time in 2001[14] and improved in 2004.[2] A standard sample of CTX-1B was provided to Dr. Jordi Molgó (CNRS), by Dr. Anne-Marie Legrand, Dr. Mireille Chinain, and the members of the Institut Territorial de Recherches Médicales Louis Malardé, Papeete, Tahiti, French Polynesia. The toxin was extracted for *Gymnothorax javanicus* moray eel liver and purified according to procedures previously described. The degree of purity was the same as that reported for determining the structure and configuration of ciguatoxin from the moray eel *G. javanicus*, based on the mouse lethality tests by intraperitoneal injection.[9,15,16]

Seven-day-old Swiss mice were obtained from the animal care facility of the University of Santiago de Compostela. Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Foetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's Modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany).

Extraction of Fish and Sample Preparation. A 70 kg specimen of *S. dumerili* and a 10 kg *S. fasciata* following the identification of Smith-Vaniz (1986),[17] were captured in April and March 2009, respectively, in waters belonging to Selvagens Islands. These Islands are part of the Portuguese Madeira Arquipelago in the North Atlantic Ocean. One section of the tail muscle of the fish *S. fasciata* (sample named f-tail) was analyzed. Four muscle sections and a liver sample were removed from the body of the fish *S. dumerili* and named head muscle (d-head), mid muscle (d-mid), ventral muscle (d-ventral), tail muscle (d-tail), and liver (d-liver). These samples were extracted and cleaned according to a similar methodology for CTXs described previously.[18] The efficiency of method was studied by analyzing the extracts discarded in each stage of the protocol, and the data

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obtained showed that there was no loss of toxin in each step. The results agree with the efficiency achieved in the method previously described (>95% for P-CTX-1B).[18] In brief, 20 g of each crude extract were cooked at 70 °C for 30 min. Then, samples were sonicated and homogenized with 80 mL methanol/hexane (3:1). The resulting homogenate was centrifuged at 4000 rpm for 20 min at 20 °C. The upper hexane phase was removed and discarded; the lower methanolic phase was filtrated though a 0.45 μm filter (Millipore Ultrafree-MC centrifugal filter units, Bedford, MA). Prior to the solid-phase extraction (SPE) with a C18 cartridge, the filtered extract was diluted in methanol/water (50:50). The cleanup was performed in four C_{18} SPE cartridges (Superclean LC-18, 1 g, Sigma-Aldrich Quimica S.A, Madrid, Spain). Each cartridge was loaded with the sample (18 mL) after being conditioned with water. Then, it was washed with 65% methanol (16 mL) and finally, CTXs were eluted with 80% methanol (20 mL). To reduce the matrix interference, the methanolic extracts were pooled and subjected to another cleanup in a silica 10 g SPE cartridge (Sigma-Aldrich Quimica S.A.). Then, a solution of 1 M NaCl (42 mL) and chloroform (67 mL) were added to the extract and it was centrifuged (2000 rpm for 4 min). The upper aqueous methanol layer was discarded and the lower organic layer was evaporated to dryness in a rotary evaporator R-200 from Büchi (Flawil, Switzerland) and dissolved in 40 mL chloroform. The cartridge was preconditioned with chloroform before loading the sample. It was washed with chloroform and then eluted with 80 mL methanol/chloroform (1:9). The resulting extract was evaporated and dissolved in methanol to be analyzed.

UPLC-MS Method. The analysis was performed in an ACQUITY UPLC system coupled to a Xevo TQ MS mass spectrometer from Waters (Manchester, UK). Chromatographic separation and identification of CTXs was achieved in a Waters Acquity UPLC BEH C_{18} column (100 mm ×2.1, 1.7 μm) equipped with a 0.2 μm Acquity UPLC in-line filter and inside the column oven at temperature 30 °C. The mobile phase for analysis consisted of two components: acetonitrile/water (95:5) (A) and water (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 50% A for 2.5 min, then, increasing to 100% A for 4.5 min. 100% A were hold for 4.5 min and reducing afterward to 50% A over 0.1 min. This proportion was maintained 0.9 min until the next run. The mobile phase flow rate was 0.4 mL/min and the injection volume was 10 μL. The MS method operated in positive ES+/ES capillary ionization mode. The capillary potential (V) was set at 2.5 kV; desolvation gas flow, 850 L/h N2; desolvation temperature, 350 °C; cone gas flow, 50 L/h N2; collision gas flow, 0 mL/min; source temperature, 120 °C; cone voltage, 50 V; collision gas, Ar at 4.5 e^{-3} mbar. All analyses were performed in MS scan and selected ion recording (SIR) mode.

The data were acquired using Waters MassLynx software and processed using the TargetLynx Application Manager. Calibration curve were obtained with CTX-3C standard. In order to calculate concentrations of compounds for which no calibration curves were generated, it was assumed that related analogues would give a similar response than CTX-3C toxin.

Cell Cultures. Primary cultures of cerebellar granule cells were obtained from cerebella of 7-day-old mice, as previously described.[19−22] In brief, cells were dissociated by mild trypsinization protocol at 37 °C, followed by trituration in a DNase solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine supplemented with p-amino benzoate, insulin, penicillin and 10% fetal calf serum. The cell suspension was seeded in 18 mm glass coverslips precoated with poly-D-lysine and incubated in 12 multiwell plates for 6−11 days in vitro (div) in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinoside, 20 μM, was added before 48 h in culture to prevent glial proliferation.

Electrophysiology. Membrane currents from single cells were studied at room temperature (22−25 °C) by gramicidin-perforated-patch recordings in voltage-clamp mode[23,24] using a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices, Sunnyvale, CA). Signals were recorded and analyzed using a Pentium computer equipped with a Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices). PClamp10 was used to generate current and voltage-clamp commands and to record the resulting data. Signals were prefiltered at 10 kHz and digitized at 20 μs intervals.

Recording electrodes were made from borosilicate glass microcapillaries (outer diameter, 1.5 mm), and the tip resistance was 5−10 MΩ. Gramicidin 10−20 μg/mL (Sigma, St. Louis, MO) was used as the membrane-perforating agent. The internal pipet solution contained (in mM): 108 Cs gluconate, 1.7 NaCl, 0.9 EGTA, 9 HEPES, 1.8 $MgCl_2$, 4 Na_2ATP and 0.3 NaGTP, pH 7.2.[25] The progress of perforation was evaluated by monitoring the decrease in membrane resistance. After the membrane resistance had stabilized (usually between 5 and 20 min after obtaining the GΩ seal), data were obtained. For perforated patch-clamp, the extracellular medium contained (in mM): 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 1.3 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES (pH 7.4).

For voltage-dependent sodium channels voltage-gated ion currents were elicited in CGCs by applying a series of 25 ms depolarizing pulses (voltage steps), in 5 mV increments, from a holding potential of −100 mV.[26] In order to calculate the ability of the different samples to block voltage-activated sodium channels, peak inward sodium currents were measured. CTX-3C was used as standard and the CTX3C inhibition of peak inward sodium currents at $V_h = -45$ mV was used to obtain a dose response curve. After a dilution 1:50 of the extract in the extracellular bathing solution, 5 μL of each extract were added to cells and the equivalent CTX-3C concentration in each sample was extrapolated after substitution of the inhibition of peak inward

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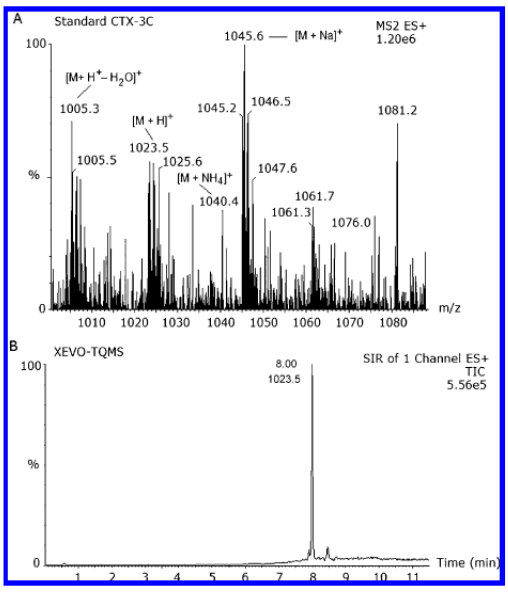

Figure 1. Mass spectrum (A) and selected ion recording (SIR) chromatogram (B) of standard CTX-3C. Mass spectrum was obtained by direct infusion and the monitored ion in SIR analysis was *m/z* 1023.5.

sodium currents on the sigmoidal dose−response curve fit obtained with the CTX-3C standard.

RESULTS

The two specimens of *S. dumerili* and *S. fasciata* captured in the Selvagens Islands in the spring of 2009, showed both positive response in an assay with Cigua Check Fish Poison Test Kit (Oceanit), developed to detect CTX in fish muscle.[13] After dissecting the fishes in several portions, sample extraction and cleanup, the CTXs profile was determinated in each sample by means of UPLC-MS method.

Before performing the analysis and in order to achieve the highest sensitivity, UPLC-MS was optimized by infusion using CTX-3C standard (500 ng/mL) in an injector system. As shown in Figure 1A, the spectrum of CTX-3C standard shows the characteristic prototype of ions formation for CTX polyethers, that is, formation of sodium and ammonium adducts and losses of water.[27] Thus, four characteristic ions were formed: ions at m/z 1023.5 because of the $[M + H]^+$, m/z 1005.5 associated with the water lost from the molecular ion $[M+ H - H_2O]^+$, m/z 1045.6 due their sodium adducts $[M + Na]^+$, and one ions with low intensity at m/z 1040.4 corresponding to its ammonium $[M + NH_4]^+$ adduct. Then, the UPLC method was optimized employing different conditions of gradient, flow and run times. After testing several alternatives, the improved parameters of the final method were described in the Materials and Methods section. One injection of CTX-3C standard at 500 ng/mL and in positive mode was made following the mass 1023.5 m/z. As it is shown in the chromatogram of the Figure 1B, CTX-3C standard was eluted at 8 min.

Once the UPLC method was optimized, the next step was to check for the presence of CTXs in these extracts from ciguateric fishes. For this, 6 samples were analyzed in scan mass mode (m/z 1000−1500). In this range of masses, it was found two prominent molecules around m/z 1040.6 and m/z 1141.6 that could be identified as CTXs. The spectrum of masses of these compounds is shown in Figure 2A and B, respectively. Each CTX produced fragmentation typical of cyclic polyether structure, including sodium, ammonium, and proton adducts of the molecular ions, and loss of water molecules, as shown in Figure 2A and B. The MS spectrum from Figure 2A showed a ion at $[M + H]^+$ m/z 1040.6 and other two intense masses because of $[M + H - H_2O]^+$ at m/z 1022.8 and $[M + H - 2H_2O]^+$ at m/z 1006.7. In addition to the protonated molecule and losses of water, the CTX gave rise to prominent $[M + NH_4]^+$ at m/z 1057.4 and $[M + Na]^+$ at m/z 1062.5 ions because of the adducts of ammonium and sodium. The MS spectrum from Figure 2B showed a high intense mass $[M + H]^+$ at 1141.6 m/z and one and two losses of water at m/z 1123.8 and 1105.8, respectively. The other two masses shown in the spectrum, $[M + NH_4]^+$ at m/z 1158.6 and $[M + Na]^+$ at m/z 1062.5, corresponding to its adducts of ammonium and sodium. Setting the masses m/z 1040.6 and m/z 1141.6 in the SIR mode, chromatograms of the Figure 3A and 3B were obtained. Each molecule, m/z 1040.6 and m/z 1141.6, gave rise to a high intensity peak eluted at the minute 6.20 and 8.19 respectively.

Searching other CTXs masses structurally characterized so far in the literature, chromatograms in mode MS scan and then in mode SIR were performed. In this range of masses, the chromatograms showed small peaks close to the masses m/z 1111.6, m/z 1061.0 and m/z 1023.5. These peaks are shown in Figure 4. The first one was eluted for m/z 1111.6 at 4.1 min (Figure 4A) and its mass spectrum is shown in the Figure 5A. This molecule was attributed to CTX-1B,[28] and this toxin was identified in 4.1 min using the CTX-1B standard. As it is shown in the Figure 5A, the mass spectrum of this toxin was dominated by $[M + H]^+$, $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + NH_4]^+$ and $[M + Na]^+$. The UPLC chromatogram following the mass m/z 1061.0 showed two closely eluted peaks with a similar mass at 5.0 and 5.6 min (Figure 4B). Finally, a peak for m/z 1023.5 at the same elution time for the standard CTX-3C was also found (Figure 4C) and its mass spectrum are shown in the Figure 5B. As it is shown in this figure, the molecule shows a mass spectral pattern typical of a polyether structure, it can observe the same ions in the same ratios like the standard, CTX-3C. Therefore, it was confirmed the presence of CTX-3C in the fish samples. Other known CTXs identified to date in the bibliography were not detected in either sample.

Once it was identified the presence of CTX in the samples, the toxin amount was quantified. The estimation of each CTX concentration in samples was determined by comparing the CTX-3C standard peak with the peak areas detected in the samples. Therefore the quantification of these toxins is provided in ng/g for each CTX in CTX-3C equivalents. For CTX-3C standard, a good seven-point calibration line among the range 5−500 ng/mL was

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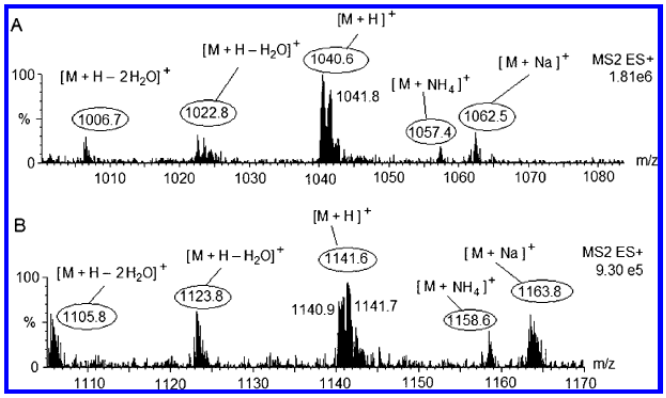

Figure 2. Positive ion spectra of the CTX analogue at *m/z* 1040.6 $[M + H]^+$ (A), and the CTX from Caribbean or Indic region with the mass *m/z* 1141.6 $[M + H]^+$ (B) from *Seriola dumerili*.

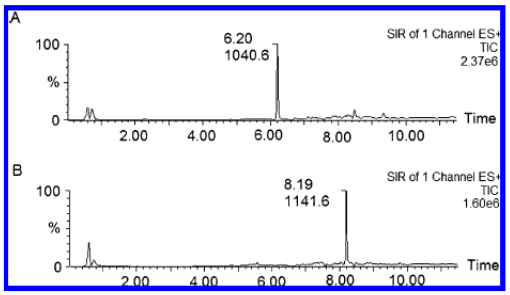

Figure 3. Selected ion recording (SIR) chromatogram of the CTX analogue (A), and the CTX from the Caribbean or Indic region (B) obtained from *Seriola dumerili* on the UPLC equipment.

obtained ($R^2 = 0.999$). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated resulting in 1.68 ng/mL and 5 ng/mL, respectively. Assuming that the different analogs have similar response factors, peak areas of the analyzed compounds show that 1140.6 *m/z* was the most abundant toxin in all samples followed by 1141.6 *m/z*. CTX-1B (1111.6 *m/z*) and CTX-3C (1023.5 *m/z*) were only present in very low levels. The CTX amount for all CTX analogs in each sample is shown in Table 1. In total, the CTX quantity in *S. fasciata* was 35.29 ng/g (measured in tail muscle). While in *S. dumerilli* the total CTX amount was: head muscle 54.35 ng/g, ventral muscle 33.29 ng/g, mid muscle 53.37 ng/g, tail muscle 53.76 ng/g, and liver 48.60 ng/g. As Table 1 shows, 4 CTX congeners were found in all fish samples except the CTX-1B which was not found neither in tail nor in head muscle of *S. dumerilli*. The LOD and LOQ in the tissue mass were calculated and the limits were estimated at 0.042 and 0.120 ng/g of meat, respectively.

To quantify the activity of the samples, the samples from *Seriola dumerili* and from *Seriola fasciata* were analyzed by an in vitro functional assay. The purpose was to quantify the presence of CTXs by perforated patch-clamp recording, a technique previously employed to evaluate the effect of CTXs on voltage-gated sodium channels.[29–31] The biological activity of the samples was evaluated by measuring its effect on peak inward sodium currents, and the amount of CTX in the samples was quantified using CTX-3C as a standard. Figure 5A shows that CTX-3C inhibited control peak inward sodium currents with an IC_{50} of 1.1×10^{-10} M (95% confidence intervals from 3.2 $X10^{-11}$ to 3.8×10^{-10} M). In this in vitro model, 5 μL of d-caudal extract inhibited control peak inward sodium current at −35 mV by 86.9 ± 3.5% ($n = 8$), which will be equivalent to the presence of 37.3 ± 0.4 ng/g CTX3C (Figure 5B). The addition of 5 μL of f-tail dilution inhibited control peak inward sodium current at −45 mV by 67.9 ± 4.8% ($n = 5$), this inhibition will give an equivalent CTX-3C amount of 40.6 ± 1.7 ng/g. Similarly, 5 μL of d-head dilution inhibited control peak inward sodium current at −40 mV by 70.5 ± 4.6% ($n = 11$), yielding an equivalent CTX-3C amount of 40.6 ± 1.5 ng/g. In the case of the d-ventral dilution, 5 μL of this sample inhibited control peak inward sodium current at −40 mV by 60.9 ± 6.1% ($n = 9$), yielding an equivalent CTX-3C amount of 45.1 ± 3.3 ng/g. Similarly, 5 μL of samples d-mid and d-liver dilutions inhibited control peak inward sodium current at −30 mV by 66.0 ± 6.1% ($n = 9$) and 82.4 ± 3.7% ($n = 8$), respectively; these amount of inhibition will be similar to the presence of 41.7 ± 1.6 ng/g of CTX 3C in d-mid dilution and 37.7 ± 0.5 ng/g of CTX-3C in d-liver dilution. The estimated amounts of CTXs in each sample, as extrapolated from the in vitro activity inhibiting peak inward sodium currents in the absence of toxins is shown in Figure 5B.

Therefore, comparable total CTXs amounts for the six tissue types were obtained by UPLC-MS method and by electrophysiological measurements. The average amount obtained in the six tissues by UPLC was 46.44 ± 3.94 ng/g while that 40.50 ± 1.16 ng/g were obtained by electrophysiological analysis. Comparable results were obtained for both techniques.

In summary, by UPLC-MS the toxin profile in samples were identified. The chromatograms shown almost two toxins belonged

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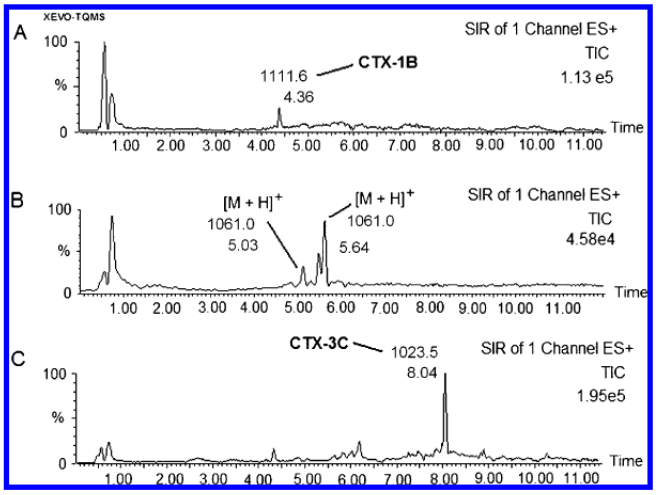

Figure 4. Chromatograms, using selected ion recording (SIR) UPLC mode of a fish sample of *Seriola dumerili*, *m/z* 1111.6 $[M + H]^+$ (A), *m/z* 1161.0 $[M + H]^+$ (B) and *m/z* 1023.5 $[M + H]^+$ (C).

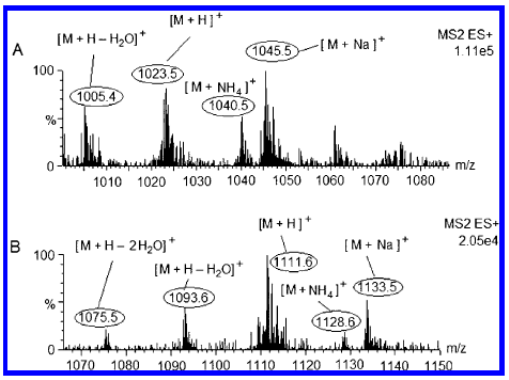

Figure 5. Positive ion spectra *m/z* 1111.6 $[M + H]^+$ (A), and *m/z* 1023.5 $[M + H]^+$ (B) from *Seriola dumerili* .

to the Pacific area, CTX-3C (*m/z* 1023.5), CTX-1B (*m/z* 1111.6), other CTX analogue at *m/z* 1040.6 and a toxin from Caribbean or Indic area the mass 1141.6 *m/z*. Both UPLC-MS and electrophysiological measurements allowed CTXs quantification in the fish tissues. These results confirm the presence of CTX in Madeira Arquipelago for the first time.

DISCUSSION

Incidents of human intoxications following the consumption of fishes attributed to CFP worldwide are appearing increasingly in countries not expected for its latitude. This issue is often attributed to climate change. However, data on how changes in temperature, salinity, etc affect microalgae are limited for assessing the impact of climate change in their communities. Substantial research is needed to evaluate the direct and indirect associations between harmful algae blooms (HABs), climate change, ocean acidification, and human health. Nevertheless, what is an obvious fact is that the average temperature of the oceans has increased in recent years and this increase will continue in the future, therefore, it is to be expected changes in the species of microalgae. A case of tropical habitats species that were found in the Iberian Peninsula coast is shown in a recent article.[32] A trumpet shell from *Charonia* family, typical of Western Mediterranean countries and from the North West Atlantic, was captured from the Southern coast of Portugal. This species was involved in a tetrodotoxin poisoning episode. The presence of palitoxin was also demonstrated in Italian[33] and Greek[34] waters. The consumption of edible fish (e.g., *Siganus spp*) was assumed to have caused CFP at other atypical site, the eastern Mediterranean.[35] In this study the presence of CTX-like substances in edible fish on the eastern Mediterranean coast of Israel was assessed. Recently, two tropical dinoflagellates of genera *Gambierdiscus* and *Sinophysis* were morphologically identified in the Mediterranean Sea.[36] The earliest record of the genus *Gambiersiscus* in the Mediterranean Sea was in Creta in 2003. Since then, Gambierdiscus cells were found on macroalgae samples (*Padina pavonica*, *Corallina elongate*, *Jania spp*,and *Cystoseira spp*) collected from 11 sites in Crete Island in summer and autumn months of the years 2003, 2004, 2005,and 2007.

There are studies that investigate how the predicted increases in temperature may affect the distribution, growth of some harmful microalgae with high incidence in the world and its toxin production.[37,38] In the case of CTXs its distribution is expected

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Table 1. Quantity of Each CTX Analogue Obtained by UPLC-MS Method for the Six Tissue Types and Total CTX Amount Obtained by Both UPLC and by Functional Method[a]

part of fish	*m/z* 1040.6	*m/z* 1141.6	*m/z* 1023.5	*m/z* 1111.6	total amount(ng/g) by UPLC	total amount (ng/g) by functional method
d-tail	39.4	7.77	0.778	<0.042	53.76	37.3
d-head	41.8	9.50	0.65	<0.042	54.35	40.6
d-ventral	28.7	2.00	0.35	0.125	33.29	45.1
d-mid	42.1	5.80	0.175	1.2	53.37	41.7
d-liver	31.7	9.50	0.55	0.4	48.60	37.7
f-tail	25.3	4.37	1.075	1.2	35.29	40.6

[a] Results are given in ng/g fish.

to shift toward higher latitudes and in fact this seems began the trend. In this study, it is confirmed the presence of CTX in the Madeira Islands. A similar case was described in 2004, in Canarias Islands,[39] also associated with *Seriola* consumption, although this article reports only the presence of C-CTX-1. However, the evidence of this change has influenced the frequency, duration and geographical extent of HABs, we consider it advisible that the influence of climate is considered and incorporated in future research involving HABs.

With this work, in addition to confirm the presence of CTX in Madeira Islands for the first time, it is shown the CTXs analogs in the fish samples. The presence of CTX-1B at *m/z* 1111.6 and CTX-3C at *m/z* 1023.5 were checked with the corresponding standards. The molecule spectrum from *m/z* 1040.6 could indicate a CTX of CTX-3C group, specially, 51-OH-CTX-3C because of 1039 molecular mass.[1] The 1140.6 is the molecular mass of different CTXs analogs, C-CTX-1, C-CTX-2, I-CTX-1, and I-CTX-2.[4] Since no standards were available, *m/z* 1140.6 molecule could be any of C-CTX-1, C-CTX-2, I-CTX-1, or I-CTX-2. In any case, it is a toxin from Caribbean or Indic area. The mass 1160.0 *m/z* could be associated with CTX-4A/B or an analogue with the same molecular weigh. But, the small amount of this molecule along with the lack of standard makes difficult its identification.

This toxin profile found in *S. dumerili* and *S. fasciata* species differs from those previously found in fish samples and strains of *Gambierdiscus spp*. For instance, in Guadeloupe, French West Indies, C-CTX-1, C-CTX-2, C-CTX-3, and 5 five new congeners of these were found in three species of fish, gray snapper (*Lutjanus griseus*), grouper (*Serranidae*), and black jack (*Caranx lugubris*);[5] I-CTX-1 and 2 at 1141.6 *m/z* and I-CTX-3 and 4 at 1157.6 *m/z* were detected in a specimen of red emperor (*Lutjanus sebae*) captured from the Republic of Mauritius;[4] CTX-1, CTX-2, CTX-3 were found in fresh fish from the Pacific Ocean in the State of Queensland, Australia;[40] and the same toxins were also identified in purified extract from the viscera of ciguateric moral eels (*Lycodontis javanicus)* collected in the Republic of Kiribati, central Pacific Ocean.[28] Finally, the main Caribbean CTX and C-CTX-1, was also identified in fish extracts of screen great barracuda (*Sphyraena barracuda)* captured from Florida Keys.[41] With regard to *Gambierdiscus* spp. in several strains of *G. toxicus* from Vietnam were identified 2,3-dihydroxy-CTX-3C, 51-OH-CTX-3C, CTX-4A/B and CTX-3C.[1] CTX-3B/C and CTX-4A/B were detected in a strain of *G. australes*.[42] The present study found in the same fish sample different CTX produced by dinoflagellates associated to the Pacific and Caribbean or Indic area. This unusual event, is not so relevant if it is taken into account that CTXs are well-known that accumulate in fish by trophic transfer in tropical fish food webs,[43] without causing the death of the fish. There was no records of fish mortalities caused by CTXs. Levels as low as 1 ng/g are believed to be toxic for humans;[44] however, this toxicity does not kill fish and therefore, do not prevent the accumulation of CTXs in fish tissue by dietary transfer. The presence of CTX in fish tissues can persist for an extended period of time and meanwhile, the fishes can migrate from the Pacific to Caribbean region or vice versa, feed in different areas, and accumulate toxins produced by several dinoflagellates.

The event of detecting toxins produced by dinoflagellates from the Pacific, Caribbean, or Indic region in the same fish is possible with CTXs. This episode is less probable in other neurotoxins even less toxic for human than CTX, for instance, the brevetoxins. The brevetoxins produced by *Karenia brevis* blooms originate in massive fish kills.[45] This death prevents the accumulation of different toxins in the fish tissue, and it also prevents the migration of fish from a sea to another one. Moreover, CTXs and their analogs, transferred through the fish food web, are bioaccumulated and may be metabolized into more potent compounds.[46] It was described that CTX-2 can be transformed in CTX-4 or CTX-3 and that themselves, CTX-4 and CTX-3 can be transformed in CTX-1.[8] Therefore, the possibility of molecular transformations is not sound due to structural similarities between Caribbean and Pacific CTXs, in fact *Gambierdiscus* may produce several CTXs depending on the water temperature. Our observations suggest that the classification of CTXs in Pacific, Indian or Caribbean is actually not useful, and should be disregarded.

The identification of toxins was performed by UPLC-MS method. This technique is a useful and rapid tool for detecting toxins, and specifically CTXs. Most lipophilic toxins are separated by LC-MS in less than 20 min,[47−49] however, with similar

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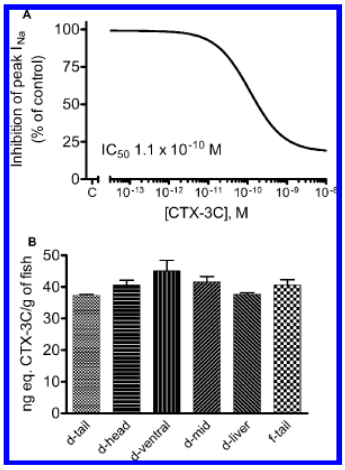

Figure 6. Effect of CTXs on peak inward sodium current in cerebellar granule neurons. A. Concentration−response inhibition of the peak inward sodium currents elicited in cerebellar granule cells by the CTX-3C standard. Values are mean ± sem from 3 different neurons. B. Extrapolated amount of ciguatoxins as evaluated by the ability of each sample to inhibit peak inward sodium currents in absence of toxin. Values are mean ± sem from 7 to 11 different neurons.

gradients, CTXs need chromatography programmes with run times exceeding 45 min.[1,41,50,51] The UPLC technique allows reducing analysis time by more than three times compared to HPLC, while maintaining peak resolution. UPLC enables a fast analysis of multiple CTXs, for this reason, it was a good choice for identifying these toxins types. By LC-MS would have resulted in significantly longer analysis time. Moreover, this is the first time that a UPLC method is developed for CTX.

To improve sensitivity and to reduce matrix effects, the samples were cleaned up following a rapid CTX extraction protocol.[18] As in previous methods of detecting CTXs, metallic impurities that lead to adduct formation were not eliminated. Ions like Na^+ and K^+ at ppm level do not affect the liquid chromatography, but make the mass spectra confused by changing the abundance of molecular ions of interest and complicate the interpretation of spectra. Despite this, a good limit of detection and quantification estimated at 0.042 and 0.120 ng/g of meat, respectively, were obtained.

In general, the results obtained by electrophysiological measurements were lower than those obtained by UPLC-MS method. Therefore, it is very probable that differences found in the quantification done by electrophysiological measurements were because of the different activities of CTX, since this technique quantified a group of toxins and not each toxin separately. Despite this, the technique only underestimated the total CTX content with respect to UPLC on a factor of 0.87.

In summary, in the present study, analytical and biological data on the presence of six congeners of CTXs in two species of *Seriola* are reported. *Seriola* spp. was previously implicated in several intoxications that occurred in Madeira Islands from 2007 through 2008. The UPLC-MS is a useful tool to confirm and identify the existence of CTX in these species of fishes and electrophysiological technique is a sensitive method for quantifying CTX obtaining comparable results to those achieved by UPLC-MS. Therefore, the results corroborate the existence of CTXs in fishes from temperate waters of the Eastern Atlantic.

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3.4. SECCIÓN III: DSP. Parámetros que afectan al nuevo método oficial de detección en la UE de toxinas lipofílicas (Reglamento Europeo 15/2011).

En la UE, así como en la mayoría de países donde existe regulación para la presencia de toxinas marinas en moluscos comestibles, el método oficial de referencia para determinar estos compuestos era exclusivamente el MBA. Aunque este método presenta una serie de problemas y limitaciones de carácter técnico, ético y legal, garantiza la protección de la salud pública. En las últimas décadas se han desarrollado varios métodos de detección para los distintos grupos de toxinas, tanto métodos funcionales cómo métodos químicos. De estos métodos, el único que ha sido reconocido oficialmente como método alternativo a los respectivos MBA para las toxinas lipofílicas a partir del 1 de julio de 2011, es la técnica de cromatografía con detección MS [164]. Actualmente hay un protocolo aprobado que está a disposición para el análisis de estas toxinas [189]. Este protocolo deja abierto a elección del analista varios parámetros que pueden modificar sustancialmente los resultados. En esta sección se estudia la importancia de estos parámetros.

A esta sección corresponden las siguientes publicaciones:

III. 1. Effect of uncontrolled factors in a validated liquid chromatography-tandem mass spectrometry method question it use as a reference method for marine toxins: major causes for concern.

III. 2. Response to Comments on “Effect of uncontrolled factors in a validated liquid chromatography-tandem mass spectrometry method question its use as a reference method for marine toxins: major causes for concern”

III. 1. El efecto de factores incontrolados en el método validado de LC-MS/MS cuestiona su uso como método de referencia de toxinas marinas.

Resumen

La técnica de cromatografía líquida acoplada a la detección por MS es el método elegido para reemplazar el MBA en la detección de toxinas marinas. Esta publicación evalúa la influencia de distintos parámetros como el solvente de las

toxinas, el método de detección de MS, la marca comercial de los solventes de la fase móvil y los equipos de detección y cuantificación de OA, DTX-1 y DTX-2. Además, este estudio compara los resultados obtenidos cuando una toxina se cuantifica con su propia curva de calibración y con la curva de calibración de otro análogo. Los experimentos se llevaron a cabo por cromatografía líquida y cromatografía líquida de ultra eficiencia con detección en tándem espectrometría de masas (MS/MS), LC-MS/MS y UPLC-MS/MS. Para este estudio se emplean tres marcas de ACNs y dos solventes de toxina y se compararon tres métodos de MS. En ambos instrumentos se comparó un método que contiene las transiciones para AZA-1, AZA-2, AZA-3, GYM, 13-desMeC, PTX-2, OA, DTX-1, DTX-2, YTX, homoYTX y 45-OH-YTX. Este método operó en modo simultáneo de ionización positivo y negativo y otros dos métodos de MS operaron en modo de ionización negativo, uno de ellos con las transiciones para detectar DTX-1, OA, DTX-2, YTX, homoYTX y 45-OH-YTX y el otro solo las transiciones para las toxinas objeto de estudio, OA, DTX-1, and DTX-2. Con independencia del equipo y de la fase móvil utilizada, la cantidad de toxina cuantificada puede ser sobreestimada o subestimada, hasta un 44 % para OA, 46% para DTX-1 y 48% para DTX-2. Además, cuando una toxina se cuantificó utilizando la curva de calibración de otro análogo, la cantidad de toxina que se obtuvofue distinta. La máxima variabilidad se obtuvo cuando DTX-2 se cuantificó utilizando la curva de calibración del OA o la de DTX-1. En este caso, la sobreestimación fue hasta 88% utilizando la curva de calibración del OA y hasta 204% utilizando la curva de calibración de la DTX-1.

En resumen, la cuantificación correcta de las toxinas DSP por detección MS depende de múltiple factores. Ya que estos factores no son tenidos en cuenta en el protocolo validado, estos resultados cuestionan la conveniencia de tener la cromatografía líquida con detección MS/MS como un método de referencia para proteger a los consumidores de las toxinas marinas. Por otra parte, la toxicidad de cada grupo se considera independiente y la toxicidad total no se suma como lo hace el MBA.

Esta publicación ha causado una gran controversia en el mundo científico y ha sido comentada por tres grupos de investigación. Estos comentarios han sido rebatidos en otra publicación por nuestro grupo de investigación.

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ARTICLE

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Effect of Uncontrolled Factors in a Validated Liquid Chromatography−Tandem Mass Spectrometry Method Question Its Use As a Reference Method for Marine Toxins: Major Causes for Concern

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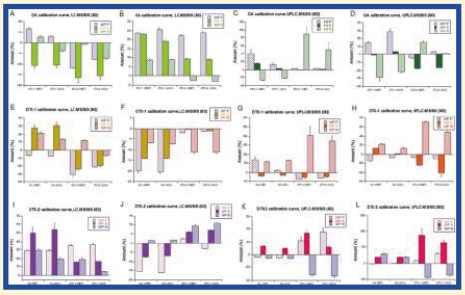
ABSTRACT: Chromatographic techniques coupled to mass spectrometry is the method of choice to replace the mouse bioassay (MBA) to detect marine toxins. This paper evaluates the influence of different parameters such as toxin solvents, mass spectrometric detection method, mobile-phase-solvent brands and equipment on okadaic acid (OA), dinophysistoxin-1 (DTX-1), and dinophysistoxin-2 (DTX-2) quantification. In addition, the study compares the results obtained when a toxin is quantified against its own calibration curve and with the calibration curve of the other analogues. The experiments were performed by liquid chromatography (LC) and ultraperformance liquid chromatography (UPLC) with tandem mass spectrometry detection (MS/MS). Three acetonitrile brands and two toxin solvents were employed, and three mass spectrometry detection methods were checked. One method that contains the transitions for azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2), azaspiracid-3(AZA-3), gimnodimine (GYM), 13-desmethyl spirolide C (SPX-1), pectenotoxin-2 (PTX-2), OA, DTX-1, DTX-2, yessotoxin (YTX), homoYTX, and 45-OH-YTX was compared in both instruments. This method operated in simultaneous positive and negative ionization mode. The other two mass methods operated only in negative ionization mode, one contains transitions to detect DTX-1, OA DTX-2, YTX, homoYTX, and 45-OH-YTX and the other only the transitions for the toxins under study OA, DTX-1, and DTX-2. With dependence on the equipment and mobile phase used, the amount of toxin quantified can be overestimated or underestimated, up to 44% for OA, 46% for DTX-1, and 48% for DTX-2. In addition, when a toxin was quantified using the calibration curve of the other analogues, the toxin amount obtained is different. The maximum variability was obtained when DTX-2 was quantified using either OA or a DTX-1 calibration curve. In this case, the overestimation was up to 88% using the OA calibration curve and up to 204% using the DTX-1 calibration curve. In summary, the correct quantification of DSP toxins by MS detection depends on multiple factors. Since these factors are not taken into account in a validated protocol, these results question the convenience of having MS/MS as a reference method for protecting consumers of marine toxins, moreover if toxicity of each group is considered independently and total toxicity is not summed anymore as it is in the MBA.



The term harmful algal bloom (HAB) is used to describe the blooms of toxin producing algae that kill fish, make shellfish poisonous, and cause numerous problems in marine coastal waters. These algae are consumed by the bivalve mollusk as a part of their natural diet. The consumption of shellfish contaminated with toxins can cause severe intoxications in human.[1] Therefore, toxins in products for human consumption are currently a major threat of global interest because of the involvement of public health and economic issues.[2] For these reasons, the search for new, rapid, and effective methods to detect marine toxins is a priority for many food safety control laboratories.

There are many classes of marine toxins. The chemical nature and physical properties allow the classification of this marine natural products into lipophilic and hydrophilic toxins. The first group includes yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs), gymnodimine (GYM), spirolides (SPXs), ciguatoxins (CTXs), and diarrhetic shellfish poisoning (DSP). Members of the DSP toxin group are OA and its derivatives dinophysistoxin-1 and -2 (DTX-1 and DTX-2). In Europe, the number of toxins to be monitored, exceeds the figure of 40. In addition to the lipophilic toxins mentioned above, hydrophilic toxins, paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) may also be present in shellfish. Nowadays, the analytical methods for detecting marine toxins comprise in vivo assays (mouse and rat

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bioassays), in vitro assays (cell, receptor, enzyme inhibition assays, and immunoassays) and chemical assays that include analysis by high-performance liquid chromatography (HPLC) with detection by ultraviolet and diode array, fluorescence, mass spectrometry, or capillary electrophoresis.[3]

The Europe Union (EU) has established legislation for 13 marine lipophilic toxins, while GYM, SPXs, and CTXs are not yet under legislation. Up until now, the reference method for lipophilic toxins was the mouse bioassay (MBA). This assay has traditionally been used to monitor toxin levels in shellfish for human consumption. For OA and analogues, AZAs, and PTXs, current EU limits in shellfish meat are set at 160 μg/kg. However, the European Food Safety Agency (EFSA) has recently published several scientific opinions on marine toxins with proposals to reduce some toxin limits and to use detection methods that could replace the MBA.[4] In this sense, biological tests are not completely satisfactory, due to the low sensitivity and the absence of specificity. Moreover, there is growing resistance against the use of animals in experiments.[5] For these reasons, the EU supports a series of methods for the detection of marine toxins as alternatives to animal testing methods, if their implementation provides an equivalent level of public health protection. These methods are in vitro assays and chemical analysis. A variety of functional and pseudo functional assays for the detection of phycotoxins have been developed over the last 20 years.[6−11] These methods are highly sensitive, have been modified to high throughput formats, and provide total toxicity response.[12] Regarding chemical assays, new analytical methods have also been developed for the determination of toxins in shellfish, especially LC−MS/MS methods.[13,14] These methods need certified standards that are not available for many of the toxins. Despite this, several studies support this approach as an essential research tool in the marine toxins field, LC−MS/MS becoming the leading and most demanded technology for improved screening methods.[15−17] A recent regulation states that LC−MS/MS-based methods are the technology to be recognized as the reference standard for the detection of lipophilic marine toxins.[18] Therefore, this technique has been evaluated in an interlaboratory validation exercise that was considered to be successful and should be applied as the reference method following indications agreed by the National Reference Laboratories Network.[19,20] After 3 years of coexistence with the mouse bioassay, the LC−MS/MS method shall become the reference method. This paper has been elicited by our concern about the stability of such a method for the control of highly toxic compounds. Although the technology is very versatile, several uncontrolled interlaboratory items such as instrumental technology, toxin standards, reagents, and approaches to perform the analysis, such as the use of toxic equivalent factors,[21] can greatly affect the final results. In this context, the present paper evaluates the influence on MS/MS measurement of the commercial source of the solvent, mass methods, toxin solvents, and instrument model on toxin quantification. Our final goal is to evaluate the solidity of LC−MS/MS as a viable alternative to the bioassay for the control of marine toxins.

MATERIALS AND METHODS

Chemicals, Materials, and Standards. Acetonitrile (ACN) was obtained from three different suppliers: Panreac (Spain) product code 221881, Sigma-Aldrich (Germany) product code 34851, and Merck (Germany) product code 10003. Formic acid and ammonium formate were from Merck. Methanol was from Panreac. All solvents employed in this work were HPLC or analytical grade, and the water was distilled and passed through a water purification system (Arium 611 Sartorius). Pure certified standard solutions of OA, DTX-1, and DTX-2 were purchased from Laboratorios Cifga (Lugo, Spain). Each ampule contained 0.5 mL of solution with 19.90 ± 0.62 μM OA, 2.10 ± 0.09 μM DTX-1, or 2.01 ± 0.09 μM DTX-2 in methanol. For UPLC−MS/MS, certified clear glass vial kit with screw cap lectra Bond PTFE/Silicone preslit 2 mL and LVI inserts with a plastic spring for 150 μL were used. For LC−MS/MS, vials and capsule with septa silicone/Teflon were employed.

LC−MS/MS Equipment. It is a combination of HPLC plus a mass detector. The HPLC system, from Shimadzu (Kyoto, Japan), consists of two pumps (LC-10ADvp), autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp), and system controller (SCL-10Avp). This system is coupled to a QTRAP LC/MS/MS system from Applied Biosystems, which integrate a hybrid quadrupole-linear ion trap mass spectrometer equipped with an ESI source. The nitrogen generator is a Nitrocraft NCLC/MS from Air Liquide (Spain).

The column used for lipophilic toxins separations was a 2 mm × 50 mm BDS-Hypersil-C8 analytical column with a particle size of 3 μm and a 10 mm × 2.1 mm guard cartridge from Thermo (Waltham, MA). The temperature was set at 25 °C. The composition of the mobile phase was water (A) and ACN/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution: starting with 30−90% B for 8 min, then 90% B was held for 3 min, reducing afterward to 30% B over 0.5 min, and hold for 5.5 min until the next run. The mobile phase flow rate was 0.2 mL/min, and the injection volume was 5 μL. Collision-induced dissociation (CID) in the ion-trap MS was performed. The electrospray ionization (ESI) source of the QTRAP was operated with the following optimized source-dependent parameters: Curtain Gas, 15 psi; collision-activated dissociation gas (CAD), 6 psi; IonSpray voltage, 4000 V; temperature, 450 °C; gas 1, 50 psi; and gas 2, 50 psi.

UPLC−MS/MS Equipment. It is an ACQUITY UPLC system coupled to a Xevo TQ MS mass spectrometer from Waters (Manchester, U.K.). The ACQUITY system integrates a column oven for high temperature and a refrigerated rack. The Xevo TQ MS mass spectrometer is an advanced, benchtop, tandem quadrupole mass spectrometer designed for high-performance LC−MS/MS applications. It employs unique T-Wave and ScanWave enabled collision cell technology to provide a highly flexible analytical tool, and it is equipped with a multimode source ESI/APCI/ESCi. The vacuum system is composed by two air-cooled Edwards Vacuum turbo molecular pumps evacuating the source and analyzer and one Varian rotary backing pump. The nitrogen generator is the same used for the LC−MS equipment.

Chromatographic separation and detection of lipophilic toxins was achieved in a Waters Acquity UPLC BEH C_{18} column (100 mm × 2.1, 1.7 μm) equipped with a 0.2 μm Acquity UPLC in-line filter and inside the column oven at a temperature of 30 °C. The composition of the mobile phase was water (A) and ACN/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation was performed by gradient elution, starting with 30% B for 3 min, and increasing to 90% B in 1.5 min. Then 90% B is held for 1 min and reducing afterward to 30% for 0.1 min. This proportion was maintained for 2 min until the next run. The mobile phase flow rate was 0.4 mL/min, and the injection volume was 5 μL. The

Xevo TQ MS mass spectrometer was operated with the following optimized source-dependent parameters: capillary potential (V) 2.5 kV, desolvation gas flow 850 L/h N_2, desolvation temperature 350 °C, cone gas flow 50 L/h N_2, collision gas flow 20 V, source temperature 120 °C, cone voltage 20 V, collision gas Ar at 4.5×10^{-3} mbar. Data were acquired using Waters MassLynx software and processed using the TargetLynx Application Manager.

Mass Spectrometric Detection. The mass spectrometers were operated in multiple reaction monitoring (MRM) detecting in positive and/or negative modes analyzing two product ions per compound: one for quantification and another for confirmation. The mass methods studied include the lipophilic toxins usually monitored in the EU. The transitions employed were DTX-1 (m/z 817.5 → 255.5, m/z 817.5 → 113.5), OA and DTX-2 (m/z 803.5 → 255.5, m/z 803.5 → 113.5), YTX (m/z 1141.4 → 1061.5, m/z 1141.4 → 855.4), homoYTX (m/z 1155.3 → 1075.3, m/z 1155.3 → 869.3), 45-OH-YTX (m/z 1157.5 → 1077.5 and 1157.5 → 855.5), AZA-1 (m/z 842.5 → 824.5, m/z 842.5 → 806.4), AZA-2 (m/z 856.5 → 838.6, m/z 856.5 → 820.6), AZA-3 (m/z 828.7 → 810.7, m/z 828.7 → 792.7), SPX-1 (m/z 692.5 → 674.4, m/z 692.5 → 444.5), GYM (m/z 508.0 → 490.0, m/z 508.0 → 392.0), and PTX-2 (m/z 876.5 → 823.5, m/z 876.5 → 213.1). Three mass methods were used to check the effect of mass method characteristics on toxin quantification. Method 1 (M1) operating in positive and negative mode checks the following toxins: DTX-1, OA, DTX-2, YTX, homoYTX, 45-OH-YTX, AZA-1, AZA-2, AZA-3, SPX-1, GYM, and PTX-2 (22 transitions for 12 toxins). Method 2 (M2) operating in negative mode checks the following toxins: DTX-1, OA, DTX-2, YTX, homoYTX, and 45-OH-YTX (10 transitions for 6 toxins). Method 3 (M3) operating in negative mode checks the following toxins: DTX-1, OA, and DTX-2 (4 transitions for 3 toxins).

Mobile Phases. To evaluate the influence of acetonitrile (ACN) in the identification and quantification of toxins, mobile phases were prepared using three different ACN from three brands. Mobile phase P (MP P) was done with ACN from Panreac, mobile phase S (MPS) with ACN from Sigma-Aldrich, and mobile phase M (MPM) with ACN from Merck.

Toxin Solvent. In order to know how toxin solvent may affect quantification, two types of toxin solutions for each concentration and toxin were made every day. OA and DTXs were dissolved in methanol or in ACN 30%.

EXPERIMENTAL PROCEDURE

The estimation of each toxin concentration was determined in its own calibration curve and also in the calibration curve of the other two standards. Calibrations were linear over the range 10−500 ng/mL using 6 points for each standard, and the linear regression R^2 values were >0.999. The proper functioning of instruments was checked everyday by monitoring key parameters, such as pressure of the HPLC pumps and retention times achieved for each toxin. In LC−MS/MS chromatograms, OA, DTX-2, and DTX-1 were eluted at 8.25, 8.73, and 9.70 min, respectively, with an oscillation in retention times lower than ±0.05 min between different days. In UPLC−MS/MS chromatograms, retention times were 2.91 min for OA, 3.08 min for DTX-2, and 3.52 min for DTX-1 with a variation lower than ±0.02 min between several analyses. Pipettes were calibrated before performing the study.

To know instrument and MS method sensitivity, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated. The LOD and LOQ are defined as the lowest concentration of toxin in a sample or specimen that could be detected and quantified, respectively, with an acceptable level of repeatability and reproducibility. For the determination LOD and LOQ of each toxin and method, a signal-to-noise ratio of 3.3 and 10, respectively, was considered from the signals obtained by 12 dilutions made by triplicate in the range from 0.15 to 30 ng/mL at concentrations of 0.15, 0.3, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25, and 30 ng/mL.

RESULTS

The aim of this paper was to design a study to determine if different usually uncontrolled parameters such as solvent brands, instruments technology, MS methods and toxin solvent affect the quantification of lipophilic marine toxins. For this, several dilutions of OA, DTX-1, and DTX-2 were measured by means of two different LC−MS/MS brand and technology systems, namely, instrument using HPLC (QTRAP system) and the faster UPLC (Xevo TQ MS system). ACNs from three different brands and two toxin solvents were also used as parameters to be evaluated.

In order to perform analysis under the same conditions and to not introduce variables other than those studied, the experiments were done by following strict guidelines. Each day we prepared 3 concentrations of each toxin (45, 160, and 320 ng/mL) in 2 solvents, methanol or 30% ACN. Then, in order to inject the same toxin quantity in both instruments, solutions were divided for LC−MS/MS and UPLC−MS/MS vials. Each result was obtained by duplicate (2 injections) and a media of both values for each experiment was considered. Altogether, 3 experiments (n = 3) were done with each mobile phase for each equipment. The mobile phases were independently prepared for each instrument everyday, and each toxin type was first quantified using its own calibration curve.

Initially, the sensitivity of three MS methods, M1, M2, and M3, was checked in the LC−MS/MS and UPLC−MS/MS systems. For this, LOD and LOQ for each toxin were calculated and results are shown in Table 1. M1 includes the transitions for all lipophilic toxins, AZA-1, AZA-2, AZA-3, GYM, SPX-1, PTX-2, OA, DTX-1, DTX-2, YTX, homoYTX, 45-OH-YTX, with the instruments operating in simultaneous positive and negative ionization mode. As Table 1 shows, OA, DTX-1, and DTX-2 LODs obtained by UPLC−MS/MS were 5.4, 7.1, and 3.5 ng/mL, respectively. Thus, good sensitivity was obtained in this system. However, when the equipment used was the LC−MS/MS, no toxin was detected even at the higher concentration used (300 ng/mL). Thus when LC−MS/MS instruments operate in simultaneous positive and negative ionization mode, the sensitivity is significantly lower. In the case of method M2, the transitions that produce a higher signal for OA, DTX-1, DTX-2, YTX, homoYTX, and 45-OH-YTX are included, therefore instruments operate only in negative ionization mode. As it is shown, toxins were detected at low concentrations in both instruments, a LOD of about 8 ng/mL for any toxin by LC−MS/MS and LODs of 3.7 ng/mL for OA, 6 ng/mL for DTX-1, and 1.6 ng/mL for DTX-2 by UPLC−MS/MS are reported. Therefore to avoid the loss of sensitivity in LC−MS/MS, it is necessary to analyze the toxins in two separate injections, one with the equipment operating in positive mode and the other in negative mode. The method M3 includes only OA, DTX-1, and DTX-2 transitions and operates in negative ionization mode. OA, DTX-1, and DTX-2 LODs obtained by LC−S/MS were 2.2, 2.3, and 2.6 ng/mL, respectively, while by UPLC−MS/MS were 1.5, 1.9, and 1.6 ng/mL, respectively. We can conclude that small LODs

Table 1. Limit of Detection (LOD) and Limit of Quantification (LOQ) of OA, DTX-1, and DTX-2 Obtained by Liquid Chromatography−Mass Spectrometry (LC−MS/MS) and Ultra Performance Liquid Chromatography (UPLC−MS/MS)[a]

		LC−MS/MS			UPLC−MS/MS		
		M 1	M 2	M3	M 1	M 2	M 3
OA	LOD	>300 ng/mL	7.7 ng/mL	2.2 ng/mL	5.4 ng/mL	3.7 ng/mL	1.5 ng/mL
	LOQ	>300 ng/mL	25 ng/mL	6.1 ng/mL	12.8 ng/mL	6.4 ng/mL	3.7 ng/mL
DTX-1	LOD	>300 ng/mL	7.7 ng/mL	2.3 ng/mL	7.1 ng/mL	6.0 ng/mL	1.9 ng/mL
	LOQ	>300 ng/mL	30 ng/mL	6.9 ng/mL	11.8 ng/mL	10.0 ng/mL	5.8 ng/mL
DTX-2	LOD	>300 ng/mL	8.6 ng/mL	2.6 ng/mL	3.5 ng/mL	1.6 ng/mL	1.6 ng/mL
	LOQ	>300 ng/mL	30 ng/mL	9.2 ng/mL	8.7 ng/mL	5.4 ng/mL	5.3 ng/mL

[a]Amounts were calculated by using three MS methods: M1, with 11 toxin transitions; M2, with 6 toxin transitions; and M3, with 3 toxin transitions.

Table 2. Effect of ACN and Toxin Solvent on the Quantification of OA, DTX-1, and DTX-2 by LC−MS/MS or UPLC−MS/MS[a]

			OA				DTX-1				DTX-2			
			Methanol		ACN 30%		Methanol		ACN 30%		Methanol		ACN 30%	
			(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)
			LC-MS/MS	UPLC-MS/MS	LC-MS/MS	UPLC-MS/MS	LC-MS/MS	UPLC-MS/MS	LC-MS/MS	UPLC-MS/MS	LC-MS/MS	UPLC-MS/MS	LC-MS/MS	UPLC-MS/MS
45 ng/mL	MP P	M2	36.9 ± 2.2	50.7 ± 1.9	42.7 ± 6.9	39.8 ± 1.1	38.8 ± 3.9	36.3 ± 9.3	**36.2** ± 4.1	40.1 ± 5.1	44.7 ± 2.1	38.5 ± 2.9	**31.5** ± 4.0	33.2 ± 2.8
		M3	61.2 ± 6.5	58.3 ± 1.6	58.5 ± 4.1	**54.8** ± 3.7	41.7 ± 3.9	**55.7** ± 6.3	40.8 ± 4.7	41.8 ± 6.3	49.2 ± 2.2	50.7 ± 3.7	**40.9** ± 3.4	42.5 ± 3.4
	MP S	M2	**27.8** ± 5.1	48.2 ± 4.7	**25.1** ± 7.9	48.3 ± 6.5	40.8 ± 2.9	28.8 ± 5.8	41.9 ± 2.6	49.8 ± 5.4	46.1 ± 3.1	**57.2** ± 0.7	51.8 ± 5.5	61.2 ± 4.8
		M3	**64.2** ± 5.7	44.3 ± 0.8	**60.0** ± 4.1	47.8 ± 3.7	47.5 ± 4.1	33.2 ± 7.6	49.8 ± 4.7	45.2 ± 6.7	55.4 ± 1.4	52.8 ± 2.5	62.4 ± 3.8	53.5 ± 3.3
	MP M	M2	38. 9 ± 6.5	56.2 ± 1.7	26.8 ± 7.5	47.8 ± 6.4	32.6 ± 3.4	37.2 ± 3.9	41.7 ± 4.7	**33.2** ± 2.2	**28.1** ± 2.8	**23.5** ± 5.5	**30.4** ± 4.3	28.0 ± 5.5
		M3	59.2 ± 6.7	**63.8** ± 6.6	47.5 ± 4.5	**26.8** ± 4.5	**33.3** ± 3.1	35.7 ± 3.25	36.9 ± 2.0	**36.7** ± 1.2	35.0 ± 2.2	24.2 ± 3.7	**36.5** ± 1.7	34.5 ± 1.0
160 ng/mL	MP P	M2	126.9 ± 17.0	153.0 ± 4.3	143.8 ± 15.7	123.5 ± 12.4	141.7 ± 11.9	149.2 ± 7.6	136.0 ± 10.2	144.3 ± 2.9	175.0 ± 8.7	193.7 ± 20.1	136.7 ± 5.7	131.7 ± 6.9
		M3	175.0 ± 12.2	176.8 ± 4.9	177.2 ± 11.9	163.2 ± 8.9	150.0 ± 10.2	140.2 ± 18.4	152.8 ±11.1	118.5 ± 17.6	184.3 ± 12.5	187.8 ± 16.8	142.8 ± 7.1	157.7 ± 4.4
	MP S	M2	141.5 ± 9.3	157.8 ± 8.1	**122.3** ± 10.3	158.2 ±10.5	149.5 ± 12.2	155.5 ± 13.3	142.2 ± 5.2	147.0 ± 7.5	156.7 ± 5.9	163.0 ± 13.3	157.5 ± 2.0	150.7 ± 8.7
		M3	**215.0** ± 12.8	150.8 ± 7.7	**200.8** ± 1.5	145.7 ± 78.3	169.3 ± 10.5	146.5 ± 14.0	165.3 ±8.4	146.5 ± 7.0	186.0 ± 14.5	195.2 ± 15.1	**198.5** ± 7.7	**193.8** ± 11.4
	MP M	M2	**116.2** ± 14.0	144.8 ± 7.3	151.8 ± 11.8	173.8 ± 6.9	**110.2** ± 3.2	139.5 ± 7.0	125.7 ± 16.7	**114.2** ± 11.1	104.5 ± 1.0	143.2 ± 6.2	134.2 ± 15.9	146.0 ± 11.5
		M3	159.5 ± 9.7	166.3 ± 11.1	176.3 ± 8.1	151.8 ± 12.0	157.2 ± 11.4	137.0 ± 9.5	122.2 ± 13.0	**85.3** ± 17.7	146.3 ± 5.2	**123.8** ± 10.9	138.8 ± 8.9	138.7 ± 6.2
320 ng/mL	MP P	M2	246.2 ± 21.5	309.7 ± 2.4	284.3 ± 25.0	313.7 ± 46.2	310.0 ± 21.1	343.7 ± 25.1	286.5 ± 32.4	**251.3** ± 41.0	284.3 ± 22.3	286.7 ± 7.9	278.7 ± 32.5	267.7 ± 32.6
		M3	334.8 ± 25.4	335.3 ± 2.4	347.3 ± 40.5	335.3 ± 2.4	315.8 ± 32.8	340.5 ± 28.0	335.2 ± 46.5	366.8 ± 11.2	301.5 ± 20.0	322.2 ± 0.8	292.8 ± 24.6	279.8 ± 19.6
	MP S	M2	258.8 ± 11.7	287.0 ± 12.0	**237.8** ± 6.7	286.0 ± 12.0	285.0 ± 27.8	249.2 ± 3.25	271.8 ± 12.0	346.7 ± 9.8	295.6 ± 6.2	346.7 ± 9.8	321.8 ± 13.6	335.0 ± 7.3
		M3	390.8 ± 6.0	303.5 ± 20.5	**375.8** ± 16.2	303.5 ± 30.5	327.7 ± 25.6	301.5 ± 17.3	330.0 ± 22.8	326.2 ± 17.7	346.8 ± 15.7	326.2 ± 17.7	366.3 ± 23.9	357.3 ± 24.7
	MP M	M2	**189.2** ± 22.2	344.8 ± 25.1	307.2 ± 25.5	315.8 ± 23.8	**196.5** ± 7.5	267.0 ± 27.5	323.0 ± 35.8	346.8 ± 43.6	201.7 ± 19.2	299.5 ± 2.0	284.3 ± 17.6	330.0 ± 7.0
		M3	331.8 ± 19.9	315.8 ± 23.8	340.3 ± 42.2	307.2 ± 25.5	304.5 ± 18.3	275.2 ± 20.75	335.8 ± 13.9	**263.7** ± 4.5	286.7 ± 1.75	**236.5** ± 2.0	290.7 ± 4.7	299.7 ± 38.9

[a]MP P: mobile phase containing ACN from Panreac. MP S: mobile phase containing ACN from Sigma. MP M: mobile phase containing ACN from Merck. Each toxin was quantified by using two MS methods: M2, MS method that includes six toxin transitions; and M3, MS method that includes three toxin transitions. Each value represent a toxin concentration obtained when it was injected 45 ng/mL OA, DTX-1, or DTX-2; 160 ng/mL OA, DTX-1, or DTX-2; and 320 ng/mL OA, DTX-1, or DTX-2 solvated in methanol or ACN 30%. Mean ± SEM of $n = 3$ experiments. The most extreme data obtained for each toxin and concentration are shown in a gray color. Values in bold represent data mentioned throughout the text.

were obtained by the methods M2 and M3 in both instruments and also by the method M1 in the UPLC−MS/MS system. The best limits were obtained with the UPLC−MS/MS instrument and the MS method that includes a small number of transitions (M3). Since the LC−MS/MS system has an extremely high LOD operating in simultaneous positive and negative ionization (M1), M2 and M3 mass methods were used in the following experiments.

Subsequently, the influence of the solvents and technology on toxin quantification was checked. Table 2 summarizes these results. First, the effect of different mobile phases (MP P, MP S, MP M) and the MS methods M2 and M3 on the quantification of OA, DTX-1, and DTX-2 were studied. Columns A, E, and I on Table 2 show the results obtained in the LC−MS/MS equipment when toxin was solved in methanol. OA quantification is shown in column A. When 45 ng/mL OA were injected and detected with the M3 method and mobile phase MP S, 64 ng/mL were quantified, while 28 ng/mL were obtained with mass method M2. When 160 ng/mL was injected, a range of 215 ng/mL (MP S and M3) to 116 ng/mL (MP M and M2) were observed. With the injection of 320 ng/mL OA, 189 ng/mL (MP M and M2) were recovered, which represents 40% less than the amount injected. Column E shows the quantification of DTX-1. Results obtained with MP P and MP S with both MS detection methods show homogeneous results close to the amount injected, although M2 results were always lower than M3 results. However with the mobile phase MP M and M2, a 35% decrease is observed at 160 and 320 ng/mL. The same reduction was observed with the same mobile phase and both methods for the lower concentration of 45 ng/mL DTX-1. DTX-2 quantification is

represented in column I. Small variability with both methods and mobile phases MP P and MP S were obtained, but again DTX-2 amounts were underestimated with MP M. This underestimation was higher, 37%, with the small concentration of 45 ng/mL, the value measured being 28 ng/mL.

Columns B, F, and J in Table 2 show the results obtained in the UPLC−MS/MS equipment when toxin was solved in methanol. OA quantification is represented in column B. These values show lower differences between results, even though with mobile phase MP M and method M3, a large overestimation was observed, since with 45 ng/mL OA a value of 64 ng/mL was obtained. DTX-1 quantification is represented in column F. Within mobile phases MP S and MP M, the amounts were slightly underestimated. However, with the use of mobile phase MP P and method M3, a 24% overestimation was observed when injecting 45 ng/mL DTX-1. Finally, column J shows the quantification of DTX-2. When 45 ng/mL DTX-2 was checked, a value of 57 ng/mL was quantified with MP S and 24 ng/mL with MP M. This means 27% more or 48% less than the amount injected. A lower amount, by 25%, is detected with 160 or 320 ng/mL DTX-2 using the mobile phase MP M and method M3. In general, the comparison of methods M2 and M3 show that results are more homogeneous, although some differences were found. In some cases, the amount of toxins quantified by the M2 method were lower than by M3, column B (160 ng/mL), column F (45 and 320 ng/mL), and column J (45 ng/mL), while in other cases the amount was higher with M2, column F (320 ng/mL), and column J (160 and 320 ng/mL). Overall, high differences in results were obtained depending on the ACN, instrument, and mass method used, even though it seems that results were more uniform using the UPLC−MS/MS instrument.

The use of mobile phase as the sample solvent is a common practice in the identification and purification of toxins.[22] Therefore ACN 30% (the composition in the start-step of mobile phase gradient) was used as the toxin solvent. Columns C, G, and K in Table 2 show results obtained in the LC−MS/MS system. OA quantification is represented in column C. When 45 ng/mL OA were injected, with the M2 mass method, 45% less was detected, while with M3 detection a 33% higher was measured. When 160 ng/mL or 320 ng/mL OA were checked with the method M2, the amounts obtained were slightly lower, whereas with M3 detection the amounts measured were quite similar to the injected, except for MP S. Column G shows the quantification of DTX-1. Results were more homogeneous, and only a small underestimation (23−15%) was observed at any concentration checked of DTX-1 with both detection methods and with the three mobile phases. DTX-2 quantification is represented in column K. When MS methods are compared, results were homogeneous except for 160 ng/mL DTX-2 with mobile phase MP S, since an overestimation of 24% is observed with a mass method of 4 transitions. However, the comparison of the effect of mobile phases shows a clear difference. When 45 ng/mL DTX-2 was injected and using mobile phases MP P or MP M, the amounts were lower than the amount injected. While at any concentration checked, the amount obtained with MP S was higher than expected.

Results of the effect of toxin solvent in the UPLC−MS/MS instrument are collected in the columns D, H, and L in Table 2. OA quantification is represented in column D. No differences were observed between results obtained by M2 or M3 detection methods or mobile phases when 320 ng/mL OA was injected. With the injection of 45 ng/mL OA, the quantity detected was 22% higher using MP P or 40% lower using MP M. In the case of 160 ng/mL OA, the highest difference was observed with MP P and the mass method within 10 transitions. Column H shows DTX-1 results. An underestimation of 23% was observed when 45 ng/mL DTX-1 was injected and MP M was used. This decrease was remarked when 160 ng/mL DTX-1 was injected, with mass method M3, since 86 ng/mL was obtained, hence a 46% less. In the case of 320 ng/mL, 22% less was obtained either with MP P (M2) and MP M (M3). DTX-2 quantification is shown in column L. As the table shows, near 30 ng/mL DTX-2 instead of 45 ng/mL was measured either with MP P and MP M, while near 55 ng/mL DTX-2 instead of 45 ng/mL was measured with MP S. An increase of 21% was observed when 160 ng/mL DTX-2 was injected with M3 detection and mobile phase MP S. No significant differences were reported at the highest concentration checked. In summary, even though several differences were observed depending on the instrument, mobile phase, and mass method being used, the results were more homogeneous if 30% ACN is used as the toxin solvent.

In summary, with the results shown in Table 2, 45 ng/mL OA can be quantified as 25 or 64 ng/mL, 160 ng/mL OA as 116 ng/mL, or 215 and 320 ng/mL OA as 189 or 390 ng/mL. The 45 ng/mL figure for DTX1 can be quantified as 29 or 56 ng/mL, 160 ng/mL DTX-1 as 86 or 169 ng/mL, and 320 ng/mL DTX-1 as 196 or 366 ng/mL. Also, the value of 45 ng/mL DTX-2 can be quantified as 23 or 61 ng/mL, 160 ng/mL DTX-2 as 104 or 198 ng/mL, and 320 ng/mL DTX-2 as 201 or 386 ng/mL. That is a ±43% variability approximately.

In order to quantify toxins when no standards are available, sometimes it is assumed that analogues from the same toxin group provide an equimolar response by MS/MS tandem detection. Thus, the calibration curve constructed for one toxin is used for quantification of other toxins from the same group. In the present study, in order to know if this approach is suitable, the quantification of each toxin was also performed using its own calibration curve and the calibration curve of the other two standards. In this case, the amount of toxin so calculated with its own standard was considered as 0%. Figure 1A−D shows DTX-1 and DTX-2 quantifications using OA calibration curves. When DTX-1 was quantified with data obtained in the LC−MS/MS instrument, an increase of 24−12% or a reduction of 22% in concentrations was reported depending on the mobile phases, mass method, and toxin solvent (Figure 1A,B). Similar increases or a 33% decrease were obtained when DTX-2 was quantified (Figure 1A,B). When DTX-1 and DTX-2 were quantified with data from the UPLC−MS/MS instrument results, a wide range of possible values were obtained (Figure 1C,D). The DTX-1 concentration could be 40% higher or 30% lower than the amount obtained with its own calibration curve, Figure 1C,D, left columns. The variation is higher in the case of DTX-2, as an increase of 90% can be observed in Figure 1C,D.

Figure 1E−H shows OA and DTX-2 quantifications using DTX-1 calibration curves. OA concentration can be 60% higher or 20% lower than values calculated with the OA calibration curve depending on the mobile phases, mass method, toxin solvent, and instrument being used. DTX-2 results obtained in the LC−MS/MS system with the mass method of four transitions are close to certified standard value, while with M2 30% less could be reported, as shown in Figure 1E,F. However, the amount of DTX-2 calculated from data obtained by UPLC−MS/MS using the calibration curve of DTX-1 show a large variability. As Figure 1G,H shows, the amount of DTX-2 could range from 40% less to 200% more. Finally Figure 1I−L shows OA and DTX-2 concentrations obtained with DTX-2 calibration curves. OA values could range

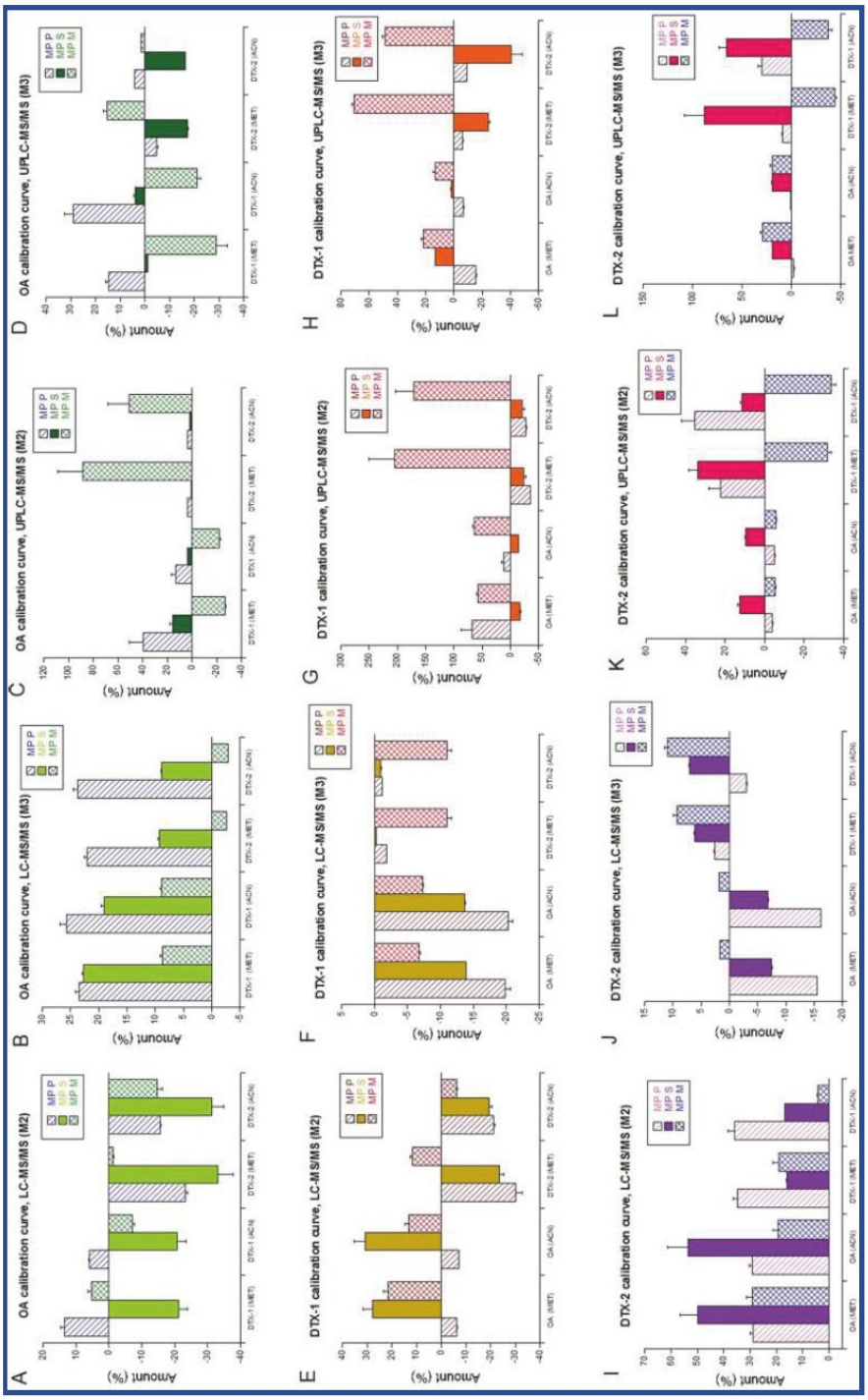

Figure 1. Quantification of DTX-1 and DTX-2 using an OA calibration curve (graphics A–D). Quantification of OA and DTX-2 using the DTX-1 calibration curve (graphics E–H). Quantification of OA and DTX-1 using the DTX-2 calibration curve (graphics I–L). Parts A, B, E, F, I, and J, data from LC–MS/MS instrument. Figures C, D, G, H, K, and L, data from and UPLC–MS/MS instrument. Terms in parentheses (MET) or (ACN) represent the quantification when toxins were dissolved in methanol or ACN 30%, respectively. Mobile phases and MS conditions are as in Table 2. Each column represents the mean ± SEM of $n = 9$ experiments. The line represents the amount of toxin obtained within its own calibration curve.

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between 50% up or 20% down with data from the LC−MS/MS system, while values from the UPLC−MS/MS instrument show lower variations, 25% increase at the most (Figure 1I,J). DTX-1 concentrations obtained with DTX-2 can be increased at 30% with data from the LC−MS/MS system, and with data from the UPLC−MS/MS the variation could oscillate between 80% higher or 45% lower (Figure 1K,L). In summary, the toxin concentration reported when the calibration curve used is not its own can be completely different depending on the different parameters.

DISCUSION

For many years, the official control method for lipophilic toxins in Europe has been the mouse bioassay MBA.[18] In this assay a sample extract was injected into the peritoneal cavity of a mouse, followed by an observation period to determine symptoms and time-to-death which usually correlates with the amount of toxin present.[15] MBA has been the most important international method for detecting marine toxins, and all analytical methods designed as official methods shall be evaluated against bioassays by internationally accepted protocols.[18] The new method, LC−MS/MS based methodology, starting in July 2011, has been recognized in Europe as the reference method for the detection of marine toxins.[18] Some interlaboratory studies involving several groups have been done to check accuracy, precision, and recovery of LC−MS/MS.[16,19,23] The EU official method has been established according to an interlaboratory validation study where different parameters about either LC or MS tandem detection had been studied but not fixed.[19] However, even in the circunstance of an approved method which had been validated internationally, several parameters are open to the criteria of the technician in charge of the analysis, such as, i.e., the commercial source of the mobile phase, while others are already approved as openly usable, such as the conversion factor on MS/MS when using one standard per group or the toxic equivalent factor, which is another source of uncertainty when providing the final result of the analysis.[21] Overall, the final result in a validated method is still affected by several uncontrolled aspects, which could provide considerable errors in the final outcome of an analysis, keeping in mind that marine toxins are rather toxic compounds.[4] In addition to these variables, the MBA detects the toxicity of all toxins together, while the chemical method detects each toxin individually, the final toxicity values of all compounds in the sample not being summed, hence allowing a far larger amount of toxin to be present in each sample. The present paper evaluates several crucial items with a relevant role in the lipophilic toxins analysis by LC with MS detection.

Some MS detection methods identify a wide range lipophilic toxins, such as OA, DTXs, PTXs, YTXs, AZAs, and SPXs, in simultaneous positive and negative ionization mode.[14,23] However, when the mass spectrometer is not capable of a fast polarity switch during analysis, the samples should be analyzed in two separate runs to avoid loss of sensitivity. In our experiments, UPLC−MS/MS was able to detect toxins in the simultaneous negative and positive mode with low LODs. However, the LC−MS/MS instrument was not able to detect the toxins using the same method at the same level of concentration. Thus, the simultaneous analysis of several marine toxin groups was not possible in this equipment. Nevertheless both instruments had comparable sensitivity with MS methods that operate only in one mode, in this case in negative ionization mode, even though lower concentrations can be detected by UPLC−MS/MS. In addition, when the MS method includes a high number of transitions, the systems lose sensitivity. In this sense, the highest ratios between LODs depending on instruments and methods reached a factor of 2.08 for OA, 1.3 for DTX-1, and 5.4 for DTX-2 comparing the same method (M2) and 5.13 for OA, 4.05 for DTX-1, and 5.4 for DTX-2 comparing different methods, in this case M2 and M3. These differences in sensitivity between instruments should be enough to detect marine toxins at an adequate level, although the analysis of contaminated samples with toxin values close to LODs can pose a detection problem. In any case, LODs obtained on our instruments with M2 and M3 methods are low enough and in the range of other LODs published for lipophilic toxins.[14,24−26] In a recent interlaboratory study, a LOD of 0.6 ng/mL for OA was reported as the average of 15 laboratories (taking into account its extraction procedure of 2 g meat/20 mL methanol).[19] In this study, some laboratories provided an LOD as low as 0.1 ng/mL of OA. These very low values are certainly surprising and mean that the participants in the exercise had an LOD far below the best LOD reported for the latest technonology available, such as quadrupole time of-flight (QTOF) or Orbitrap-based mass spectrometers;[25] even so, for these technologies the best LODs reported are 2.8 ng/mL of OA.[26]

In addition to the sensitivity, the MS method selected has a relevant role on the quantification of toxins. Independent of the mobile phase used, OA, DTX-1, and DTX-2 amounts analyzed by LC−MS/MS using method M2 (10 transitions) were considerably lower than those obtained by the method M3 (4 transitions). The underestimations were up to 40% for OA, 39% for DTX-1, and 37% for DTX-2 when M2 instead M3 was used as the MS method. With the UPLC−MS/MS, instrument results were more homogeneous when 10 or 4 transitions were used. An underestimation of 20% approximately is sometimes observed with M2 with respect to M3, but occasionally the subestimation is observed with the M3 method with respect to M2. Therefore, a previous specific setting of the number of transitions in the MS detection method is very important in order to reduce variability.[27]

Different results were obtained according to the solvent brands employed, and the heterogeneity was also higher with LC−MS/MS equipment. It may be possible that the differences in quantification can be due to the impurities of each ACN since each brand has different composition. The OA amount detected with mobile phase MP S was bigger than the amount injected, up to 42%, while the amount of DTX-1 and DTX-2 detected with MP M were lower than the amounts injected. However, even for the same ACN, the content of impurities could be different depending on each lot of product. In order to validate a detection method, Commission Decision 2002/657/EC shows that the accuracy with 10 μg/kg analyte levels should be between −20% and +10%.[23,28] Therefore, the variability found in the analysis is outside of the permitted limit for the validation of this technique as an official method.

Pure or diluted methanol is usually employed to disolve the lipophilic marine toxins due to its property of conferring stability to the toxins.[29] Besides methanol, the use of mobile phase as a sample solvent is also commonly employed in identification and purification of toxins.[22] In order to know which organic compound is more suitable to dissolve the toxins, this study compared the results obtained in methanol and 30% ACN. In the LC−MS/MS system, if OA was dissolved in methanol and quantified, an overestimation of 42% was obtained for 45 ng/mL; however, the overestimation achieved for the same concentration in ACN was 33%. Therefore, overestimation for OA is lower if dissolved in 30% ACN than in methanol. In both solvents, DTX-1 amounts were underestimated using MP M and method M3. When

DTX-1 was dissolved in methanol, the amount obtained was 39% less, while being dissolved in ACN 30% the amount was 24% lower. Therefore, again DTX-1 was also less underestimated in ACN 30% than in methanol. Equally, results obtained for DTX-2 were slightly less underestimated when toxin was dissolved in ACN 30%. In methanol, the maximum underestimation was 37%, while it was 32% when toxin was in 30% ACN. Similar results were obtained by UPLC−MS/MS. In summary, although similar variability is shown, in general more exact values were obtained with ACN 30%. In any case, since a high variability was observed between solvents, it is necessary again to emphasize the importance of setting the toxin solvent to be used. In this context, different solvents are reported in validated and official extraction procedures for MS quantification of lipophilic marine toxins.[16,19,20] A major source of results variability will therefore be the change of solvent in a given laboratory.

Since standards for all toxins are not available, the calibration curve of one toxin standard is often used for quantifying other toxins from the same group,[19,20] assuming a given ionization conversion factor. This important issue was also evaluated, and no comparable results were obtained. The lowest variability was achieved when OA was quantified by DTX-1 or DTX-2 calibration curves. This quantification type is unusual since the OA standard is commonly available. However, when DTX-1 was quantified in an OA calibration curve, the amount obtained was up to 40% higher or down to 25% lower depending on the ACN and equipment being used. DTX-1 concentration was also very different when it was quantified in a DTX-2 calibration curve. In this case, the amount obtained was 88% higher or 44% lower depending on the conditions employed. Finally, the highest variability was obtained when DTX-2 was quantified using either an OA or a DTX-1 calibration curve. The overestimation was 88% with the OA calibration curve and up to 204% using a DTX-2 calibration curve. With these values, a sample containing 150 μg/kg of any of these toxins, considered as negative by EU regulations[30] can be quantified as 210 or 112.5 μg/kg in the best cases or 456 μg/kg in the worst case. If a sample contains 40 μg/kg, therefore being negative for monitoring purposes, with the theoretical limits suggested on the EFSA opinion,[4] it would be quantified as 121 or as 30 μg/kg depending on the experimental conditions. On the other hand, a sample with 287 μg/kg (positive for EU legislation and rather toxic) would be detected as 155 μg/kg, hence negative, under certain experimental conditions. These extremely different results are due to the lack of ruggedness of this method. In this context, several studies use calibration curves with six standards over the range 3−50 ng/mL.[14,19] This extremely short dynamic range in addition to the use of nonappropriated standard calibration curves means a high probability of imprecise and nonaccurate results when chromatography with MS/MS detection is used to quantify lipophilic toxins. This variability questions the approach taken, at this time, by the EU to rely on MS/MS as a reference method for monitoring marine toxins for food safety protection, since the rate of false positives or false negatives might be unpredictably high for values close to the legal limits, being of special concern those false negatives, where toxic samples might be identified as negatives with a given combination of parameters that are not covered by current validation protocols. In addition, the new legislation allows each toxin to be quantified regardless of the presence of other toxins, while the MBA detects all toxins together, hence the legal toxic value is potentially increased several fold with the chemical approach when several toxin groups are present in the sample. It is very important to keep in mind that this paper only provides information about the variability on a few parameters and toxins under very controlled conditions and with no matrix effects. In real conditions, with matrix effects, cooking, and more toxins being analyzed in a single injection, the final analytical results would become only more unpredictable. Given the fact that the toxic equivalent factors would be needed for the correct translation of analytical values into toxic levels, the final uncertainty of MS/MS as a reference method is very high. The EFSA opinion on OA and analogues[31] does suggest that bioassay provides an uncertainty of 40−50% to detect OA-group toxins at the current EU legal limit. However, this figure is not improved by MS/MS, if all the analytical variables are not taken into account. This work demonstrates a variation of up to 200% using MS/MS, and yet matrix and TEF are not included in this work, hence the reliability of MS/MS by no means justifies this approach as a fair reference method.

Collaborative studies supply information on standards needed for the exercise, the parameters in MS, or chromatographic methods and the transitions employed for toxins quantification.[19,20] However, as it was shown, there are other key factors that affect the quantification, as the number of transitions employed in the MS method, the toxins solvent and solvent brands, or the standards used that are not fixed. From these results, in addition to these guidelines and due to the lack of ruggedness of the method, a single-laboratory validation should be regularly programed if some of these items are changed. If the variability in one laboratory is high, the interlaboratory method validation with open options is a difficult task. In summary, analytical methods are useful tools to show the toxin profile, to characterize new toxins, or to search for its analogues, but in order to protect public health and food safety, more tight conditions, in order to increase methodology reliability, are needed.

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analytical chemistry

COMMENT

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Response to Comments on "Effect of Uncontrolled Factors in a Validated Liquid Chromatography−Tandem Mass Spectrometry Method Question Its Use as a Reference Method for Marine Toxins: Major Causes for Concern"

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This is a response to the three comments on the recent article in *Analytical Chemistry*, from Gago-Martinez et al., Gerssen et al., and Holland et al. The basic objection is that not adequate quality controls were followed. We deny this argument. Holland et al. base their entire comment in a claimed carry-over effect that we show does not exist. In addition, they support their reasoning with the argument of a plot they made up from our summary data. Gago-Martinez et al. claim the protocol we followed is not the EU-RL, and we show it not to be the case. Gerssen et al. pose questions about the article that we clarify. We find the main position of the three responses remarkable: because they do not agree with our data, their conclusion is that our results cannot be true and question our work as biased data.

The manuscript shows data obtained by the LC−MS/MS procedure to detect and quantify lipophilic toxins. The scientific design of the experiments produced solid data, which were reviewed by experts. A long Methods section was first proposed; however, because of the lack of space it was reduced to fit the printed space in the journal. Strict QA/QC procedures were followed throughout the paper. The proper functioning of the instruments was checked everyday; the apparatus was first calibrated with its own calibration protocol and periodically reviewed. The sequence of injections (first lowest concentration), calibration solutions, times of retention, peak resolution, peak definition, correlation coefficient, variation between calibration slopes ≤25%, or any other parameter were carefully controlled. Mass parameters (dwell times, collision energy, voltages, temperature, etc.) were first optimized in each equipment, and the same parameters were used for the three toxins studied. The experiments were carried out for 2 months using full equipment time, which means no other measurements different than those in the experiments for the manuscript were done during that period. Blanks were injected between each sample, and the signal intensity was always controlled (LC−MS/MS equipment = 4.1 ± 0.5 cps and UPLC−MS/MS = 55.5 ± 6 cps). No carry-over signal was ever observed. The calibration curves of each toxin ($r > 0.99$) were done with pure certified standard solutions. In summary, to avoid false conclusions, all of the parameters that the comment manuscripts of Holland et al. and Gerssen et al. had referred to were carefully controlled. We have a long and successful experience using this technology, and because of that we designed this paper to study uncontrolled as well as not usually defined parameters. LC−MS/MS technology is a sensitive and powerful analytical methodology that should be carefully used if to be applied as a routine method by many laboratories. The point is not the methodology, the question is always food safety, and if false positives by a mouse bioassay were a problem, false results by uncontrolled LC−MS/MS can be a bigger problem. It is necessary to keep in mind that all this discussion is referred to the use of LC−MS/MS with just three known toxins, the detection of other unregulated toxins (palytoxin, ciguatoxin, tetrodotoxin, cyclic imines) is still not resolved with this kind of analysis although it was somewhat controlled with the mouse bioassay.

COMMENT: GAGO-MARTINEZ ET AL.

The experiments were performed following the "EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in mollusks by LC−MS/MS, version 2, July 2010". The new version of this SOP, version 3, was published in May 2011 and the last one on July 2011, version 4 (the paper was submitted to the journal in March 2011).[1] It is noteworthy that the changes in these new versions include suggestions brought about by our paper. The method used in our paper is the one proposed by the EU-RLMB, by choosing the sections 4.2.1 acidic chromatographic conditions, 4.3. reference materials okadaic acid standard solution, DTX1 and DTX2 standards were also used, 7.1.1 LC−MS/MS conditions (chromatography under acidic conditions), 7.2. mass spectrometry detection: fragmentation conditions with negative ESI detection, and 8.1. calibration curve and sample injection. A higher dynamic detection range was used by us with no matrix. These are theoretically easier conditions than those with a matrix. The SOP published by the EU-RLMB propose three different chromatographic conditions, different columns, and 24 examples of transitions and MS/MS fragmentation conditions. The dynamic range proposed by the SOP is very short and defined only as an example, which is not only unclear but rather unprecise. It is striking that current EU-RLMB SOP not only recommends a specific commercial

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source of standards, it actually recommends commercial lots of those standards, which does not sustain an SOP based on robustness. This is rather important, as the method is intended to be used for commercial international trading and also for legal purposes. The use of proper methodology, modifying the unfixed parameters (solvent brand, standard, or number of transitions) shows evident differences in the results.

SOPs section 8.3, quantification, specify that the calibration curve constructed for OA is to be used for the quantification of dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2), assuming an equimolar response, page 4 of SOPs, versions 2, 3, and 4. Again, this assumption originates many errors for quantification, and certified reference standards for each toxin should be used.

The conclusion that our paper did not follow QA/QC procedures like those stipulated in the approved method is unsustained since we strictly followed the protocol and we studied the parameters that were not fixed by the SOP. It is therefore evident that "for determination of lipophilic marine toxins" the protocols should be better defined in order to detect and quantify marine toxins and to protect human health. In fact some of these technical issues were found important by the EU-RLMB in the SOP version 4, Annex B, hence incorporating instructions based on our paper.

Finally, as the EU-RLMB is linked to DG SANCO, it is a matter of further thinking the fact that, as we point out in the article, the mouse bioassay had an absolute limit of 160 μg/kg AO equivalent toxicity in 24 h, covering all lipophilic toxins, and current regulation 15/2011[2] requires LC−MS to be "not less effective than the biological methods and that their implementation provides an equivalent level of public health protection". It is certainly surprising that all three authors seem to show their discomfort with our main conclusion, "major causes for concern", but defend entusiasthically a method that by no means "provides an equivalent level of public health protection", since LC−MS detects only targeted toxins. Moreover, protocol is designed to identify each toxin group independently, hence allowing different toxin groups to reach, in the same sample, up 160 μg OA and analogues, plus 160 μg of AZA, plus 1 mg of YTX, not to mention very lethal toxins (tetrodotoxin, ostreocins) which are not even analyzed for now and were at least picked by the bioassay.

Finally, we wish to show our surprise and concern for the position of the authors signing the comment, as the text in it refers to internal SOPs of the European NRLs network, and the so-called AOAC Marine Toxins Task Force or the U.S. FDA has no link with the function of the EU-RLMB or its Web page.

COMMENT: GERSSENET ET AL.

In the page 5909, line 17, we cite reference 19 instead of 20. We are sorry about that and apologize to these authors.

Appropriated dwell times were used, UPLC−MS/MS, 0.078 s (method 4 transitions) and 0.028 s (method 10 transitions) and LC−MS/MS total scan time 1.1 s (method 4 transitions) and 1.05 s (method 10 transitions). When the equipment was used simultaneously in positive and negative, the LC−MS/MS total scan time was 1.11 s for the negative and 1.18 s for the positive (24 transitions) and UPLC−MS/MS dwell times were 0.015 s for the negative and positive. With these parameters and in our LC−MS/MS detector (QTRAP 2000), no simultaneous detection was possible. This is a known problem for the use of unsophisticated MS/MS detectors, and it is actually the reason why we chose this equipment, to compare technologies from two generations.

The same settings were used and fixed in each equipment for the three toxins detected. In Figure 1, when a toxin is quantified against another one, an overestimation was observed, and when the second is quantified against the first, an underestimation was observed. This is the case in 62 out of 72 quantifications, which leaves 10 data out of this pattern, well within statistical variation, since the amount of toxin was calculated with their own standards and considered as 0%. Therefore standards of each toxin should be used to perfectly quantify the toxins.

In their comment, they first state that the paper contains results of experiments where technical errors were made. However, throughout text, only questions are done about how the experiments were performed. We are surprised to conclude that they are questioning the way we work rather than the experiments. Lets just say that experiments were done with adequately performed and optimized methods. The conclusion that the authors concerns are not convincing is out of place, since we just provide our data and findings. Their questions and concerns that point that our work has inconsistences are the consequence of an unfit EU-RL SOP that we followed. LC−MS/MS is a good analytical methodology that should be carefully used under controlled conditions if it were used "to determine" marine toxins in human food.

COMMENT: HOLLAND ET AL.

Incidents of human intoxications in European countries after consumption of fish and mollusks attributed to marine toxins are not uncommon. LC−MS/MS methods are not suitable to detect new toxin analogues, and this lack contributes to a public health hazard. European legislation covers gasteropods, and we have reported massive amounts of tetrodotoxin as a cause of human intoxication.[3] Likewise ciguatera has been identified as a major threat in Europe.[4]

We fully understand the international effort done in the LC−MS/MS field and also in other alternative detection methods, since we have a well-known part of this international effort. We do not agree with the claim that the LC−MS method for marine toxins has been developed in Canada, as this has been a formidable work by several other laboratories worldwide who provided major contributions to current methods. We understand that if the international community is moving to a new detection method, this method should be perfectly controlled and should guarantee a correct detection since the control of marine toxins in seafood is the point. The authors totally miss this point: it is not about LC−MS/MS methodology but if the LC−MS/MS SOP can guarantee food safety. Palytoxin analogues were detected in mussels,[5] and LC/MS is at this time not capable of dealing with this problem, not to mention that they are not covered by legal requirements, like ciguatoxins or tetrodotoxin.

It seems that Holland et al. did not fully understand Figure 1, and therefore they have drawn the wrong conclusions from the paper. This explains several odd comments and the wrong meta-analysis performed, which is not possible with the 18 plots they obtained from the averages we reported. The increase in the amount of detected toxin over the amount injected when 10 transitions were used was obtained at the same time than the decrease observed when 4 transitions were used. If the approach is wrong in the first case it is also wrong for the second one.

Therefore if carry-over happens in the first case, with wrong results as they claim, they must use a contradictory reasoning if trying to explain the decrease observed with the other method. Our point is that this is a weakness of LC−MS methodology, as has been recognized in other analytical fields, and not a lack of QA/QC or the experimental work.

Sample carry-over is a major problem that can influence the accuracy and precision of LC−MS/MS results. It is caused by residual analyte from a sample analyzed earlier in the run. When blanks or low-concentrations samples follow high-concentration samples, there is a potential risk of contamination and carry-over. There are two types of LC carry-over: column carry-over and autosampler carry-over. The first is observed as an elevated, downward-drifting baseline in a blank sample analyzed after high concentration samples. We injected a blank solution after each sample injection, and no carry-over was observed (blank analysis = 4.1 ± 0.5 cps and 55.5 ± 6 cps). The other type is the autosampler carry-over. It is associated with the interaction of an analyte with the flow path components of the system. One way for this to occur is by rinsing. We used the recommended wash solutions, composed of methanol−water. Our sample injections always followed the same sequence, from low to high: first, 45 ng/mL, followed by 160 ng/mL and finally 320 ng/mL. In addition, in the calibration curves, the lowest concentration was injected first. Authors unsuccessfully tried to demonstrate that the overestimation observed in M3 results are due to carry-over, and they explain the underestimation observed by using M2, 10 transitions, as a QTRAP bad optimization. This line of argumentation is very weak: the carry-over is a chromatographic problem, not a detector problem. It is not possible to have carry-over with one method and not with another one that uses the same chromatographic conditions. The only difference between both methods is the number of transitions. In summary, the major complaint about our experimental conditions and carry-over is unsustainable.

Holland et al. have used data from three calibration curves to create one with three points, $y = 0.9952x + 16.2$, $R^2 = 1$, that shows an overestimation. We obtained 54 plots per toxin and method, and with these results we elaborate on our conclusion. The authors should know that to analyze our data, they need the original data, as Table 1 is a summary, and not use a few results they chose from the paper to draw conclusions.

We are glad the authors agree with our claim that each toxin should be identified with its own standard. This is now possible since high-quality standards are available in Europe (CIFGA). However this important issue was not highlighted in the SOP form EU-RLMB that we tested in this paper. In that SOP, an equi-molar response is assumed and the OA calibration curve is used for the quantification of DTX-1 and DTX-2. Therefore the question that authors point as a nonissue is a crucial subject that only recently has been introduced after the publication of our paper. We agree with the statement that LC−MS in regulatory laboratories must be accompanied by rigorous quality control procedures, hence it is very important to fix and control all the items that are not well-defined in the EU-RLMB protocol to be used by any official laboratory.

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4. Discusión

Las CTXs y los SPXs son dos grupos de toxinas lipofílicas que cada vez presentan una mayor distribución a nivel mundial y para las cuales la UE no presenta métodos oficiales de detección. Pero la situación particular de cada una de ellas es distinta. Los SPXs son muchísimo más frecuentes en las costas europeas, sin embargo, al no registrarse casos de intoxicaciones humanas, de momento no se contempla su regulación. Estas toxinas aparecieron por primera vez en Canadá [118,119] y a partir de ahí se han ido extendiendo a distintos países de todo el mundo, incluyendo USA (golfo de Maine) [125], Dinamarca [122], Italia [126], España [127], Francia [128], Irlanda [129], Escocia [130], Noruega [131] y Chile [132]. Hoy en día, el grado de expansión es tal, que es difícil comprar en el mercado mejillón sin que lleve trazas de estos compuestos [152]. Por otra parte, las CTXs son típicas de los océanos Pacífico e Índico y zonas del mar Caribe [108,109]. En los últimos años, estas toxinas están aumentando puntualmente en países en los que no es habitual su presencia debido a su localización geográfica [115], como es el caso del mar Mediterráneo [115,207] y océano Atlántico que incluye las islas Canarias [114] y el registro del archipiélago de Madeira mostrado en la presente tesis doctoral [208]. Las CTXs presentan una alta toxicidad [9] y pueden llegar a suponer un elevado riesgo para la salud de los consumidores en cantidades muy pequeñas, ya que se ha visto que niveles de 1 ng/g son tóxicos para humanos [101].

La importancia del estudio de SPXs presentada en esta memoria de tesis se debe a varios factores. Estos compuestos son el grupo más grande de las CIs y uno de los mejor caracterizados estructuralmente, sin embargo, la información relativa a su toxicidad es muy limitada. Los SPXs no parecen provocar efectos tóxicos aparentes en humanos [209], pero su mecanismo de acción sugiere que pueden ser un riesgo a largo plazo para los consumidores, ya que son potentes antagonistas de los mAChR y los nAChR [210]. En caso de que se regulen, se necesitarán métodos de detección rápidos, sensibles y específicos. Por lo que hay una necesidad importante de buscar métodos para su detección. Hoy en día, para su monitorización en moluscos, se suelen incluir en los métodos de LC-MS/MS junto con el resto de las toxinas lipofílicas que sí que están legisladas [163,195]. Para poder llevar a cabo los estudios químicos y toxicológicos de cualquier toxina se necesitan patrones, cuya disponibilidad en el mercado es escasa o nula para muchas de ellas. Estos materiales de referencia se pueden extraer y purificar a partir de moluscos bivalvos intoxicados naturalmente [211], pero para ello se debe

esperar a la aparición de un evento tóxico de forma natural, y además los extractos de moluscos precisan de muchos pasos de purificación. Una solución consiste en el aislamiento y cultivo de las especies de microalgas productoras de las toxinas y otra en obtener los estándares de toxinas de forma sintética. La síntesis de los SPXs es un proceso complejo y hasta la fecha, ninguno de los 14 SPXs estructuralmente caracterizados ha sido totalmente sintetizado. Solo se ha realizado la síntesis del grupo bispiroacetal 5,5,6 de los SPXs B y D [139,212]. Por lo tanto, en el caso de los SPXs parece que la mejor opción es la producción a partir de cultivos de *A. ostenfeldii.* Para poder conseguir cantidades suficientes de SPXs y en poco tiempo, son necesarios estudios experimentales de optimización de la producción a partir de los dinoflagelados. En este sentido, en la presente tesis doctoral se estudiaron algunos factores que pueden influir en el crecimiento y producción de SPXs por el dinoflagelado *A. ostenfeldii* [213]. Este dinoflagelado produjo dos SPXs, 13-desMeC y 13,19-didesMeC, pero en proporciones distintas dependiendo de la salinidad, medio de cultivo y fotoperíodo. Lo importante de este estudio fue conocer en qué condiciones *A. ostenfeldii* produce SPXs y si la producción es la misma, en cuanto cantidad y tipo, al variar los parámetros que influyen en el crecimiento. El siguiente paso para obtener los estándares de SPXs fue el desarrollo de un método para purificar grandes cantidades de estos compuestos a partir de los cultivos de *A. ostenfeldii* [214]. Estos dinoflagelados produjeron, además, toxinas pertenecientes al grupo de las PSP, por lo que el protocolo se diseñó con varios pasos de extracción y limpieza y varias particiones con disolventes orgánicos con el fin de separar los 2 grupos de toxinas. Finalmente, se obtuvieron ambos compuestos con alta pureza, 97% para el 13-desMeC y 99% para el 13,19-didesMeC con una buena recuperación, repetitividad y estabilidad. En definitiva, el cultivo de *A. ostenfeldii* en grandes volúmenes permitió la producción de SPXs en cantidad suficiente para llevar a cabo con éxito su extracción y purificación y a su vez, con el protocolo de purificación desarrollado se consiguieron grandes cantidades de compuesto puro que fue utilizado como patrón en los siguientes estudios de esta tesis, tanto en los métodos químicos (LC-MS/MS) como en los estudios funcionales y de toxicidad.

El siguiente paso fue el estudio de un método de detección funcional adecuado para utilizarse en el control de SPXs [215]. Los métodos funcionales aportan información global de la toxicidad, pero no describen cuántas toxinas y en qué cantidad pueden estar presentes en una muestra. Los métodos químicos, permiten la identificación y cuantificación de las toxinas por separado pero presentan el

inconveniente de que necesitan patrones y no informan sobre la toxicidad. Cada método tiene sus ventajas e inconvenientes y en última instancia es responsabilidad de los expertos decidir cuál es la tecnología más adecuada según la situación particular de cada toxina. Muchas veces el no conocer cuál es la diana del compuesto dificulta el diseño de un método funcional basado en el uso *in vitro* de su receptor específico [216]. Este no es el caso de los SPXs ya que hay varios estudios que demuestran que bloquean los mAChR y los nAChR [3,137]. Por lo tanto, utilizando los nAChR, se diseñó un método directo para la detección y cuantificación de estos compuestos en muestras de mejillón utilizando la técnica de la FP. Este ensayo es reproducible, simple y sensible, capaz de cuantificar 13-desMeC en el rango de 50-350 µg/kg de carne [215]. Este método ha resultado en una mejora de sensibilidad, rapidez y sencillez comparado con el anterior método de inhibición para detectar espirólidos [196]. El método directo presenta un LOD de 50 µg/kg 13-desMeC mientras que el LOD del método de inhibición es de 85 µg/kg de 13-desMeC. Otra ventaja es que solo precisa 10 min de incubación frente a 2h y 30 min y no requiere la alpha-bungarotoxina equitetada con el colorante Alexa Fluor 488, con lo que da lugar a en un método más sencillo.

Los estudios de toxicidad *in vivo* de SPX aportaron información farmacocinética de los SPXs en animales, necesaria para entender y mejorar el conocimiento sobre la toxicidad oral e i.p. de estos compuestos y sus efectos toxicológicos. La falta de estos datos previene el establecimiento de límites legislados para los SPXs y para las CIs en general. El estudio de toxicidad realizado en la presente tesis doctoral [217] es una descripción detallada de los síntomas observados en el ratón para tres SPXs, 13-desMeC, 13,19-didesMeC y 20-MeG y revela que los SPXs se detectan en sangre, orina y heces en diferentes tiempos, es decir, muestra la farmacocinética de una toxina no regulada después de la administración oral. Cualquier aportación en este campo es muy valiosa para la posible regulación de estas toxinas. De hecho, EFSA tuvo en cuenta algunos de estos resultados de este estudio de toxicidad para evaluar el riesgo en la salud humana respecto al consumo de SPXs [202].

En resumen, el grupo de los SPXs representó un área de investigación relevante en esta tesis doctoral tanto en el sentido toxicológico como químico. El interés de su estudio se debe a que es una toxina de nueva aparición y cada vez más frecuente en nuestras costas, tóxica para el ratón aunque no se hayan registrado casos de intoxicaciones humanas. Además existe una gran controversia en sobre si esta

toxina debería o no estar legislada. Para que esto ocurra se necesitan datos y nuevos estudios de toxicidad que se tienen que realizar con estándares de estos compuestos. La mayor parte de las toxinas no están disponibles comercialmente con lo que si se quieren usar para investigación, es necesario sintetizarlas o producirlas.

Las CTXs son producidas por especies del género *Gambierdiscus* y como son típicas de aguas tropicales, el hecho de encontrarse en aguas cercanas a las costas europeas se atribuye al cambio climático [9]. Lo cierto es que los datos de cómo los cambios en la temperatura, salinidad, etc, afectan a las microalgas son limitados para evaluar el impacto del cambio climático en los episodios de HABs. Pero un hecho obvio es que la temperatura media de los océanos ha aumento en los últimos años y este aumento continuará de aquí en adelante por lo que se esperan más alteraciones en las especies de microalgas. Estos cambios no sólo se aprecian en la presencia de CTXs producidas por *Gambierdiscus* [114,115,208] sino también en otras toxinas de climas tropicales como las TTXs y las PlTXs. En este sentido, intoxicaciones debidas a las TTXs son frecuentes en países asiáticos debido al consumo de pez globo o pequeños gasterópodos contaminados [218]. Sin embargo, una caracola del genero de *Charonia,* de hábitats tropicales, se encontró no hace mucho tiempo en las costas de la península Ibérica [36]. Esta caracola, que fue capturada en el sur de Portugal, dio lugar a un episodio de envenenamiento por TTX [36,219]. Las PlTXs son producidas por dinoflagelados del género *Ostreopsis* [220]. Este dinoflagelado que también es típico de aguas tropicales, se detectó por primera vez en aguas templadas del Mediterráneo en la década de los 70 [221] pero fue en los últimos años cuando se han registrado las grandes HABs en la costa mediterránea de Europa y norte de África, en la costa atlántica de Portugal [222,223] y también en España [224]. Las CTXs aparecieron por primera vez cerca de las costas europeas en el 2005 [114] y aún no se han registrado muchos casos, pero teniendo en cuenta estos antecedentes, los expertos consideran que el incremento de la temperatura de los océanos ocasionada por el cambio climático dará lugar a un aumento de la frecuencia de aparición de las CTXs en Europa. En este sentido, la aparición de CTXs en Madeira que se describe en el presente trabajo apoya la teoría de la contribución del cambio climático a la distribución de estas toxinas. Pero también hay que tener en cuenta que poco se conoce sobre la distribución y diversidad de las especies de *Gambierdiscus*. Se sabe que el perfil de

CTXs en los peces depende considerablemente de la región y de los organismos causantes y que existen diferencias entre las CTXs producidas por distintas cepas de *Gambierdiscus* [199]. Durante muchos años se pensó que estos organismos solo existían en áreas del Pacífico, del Caribe o del Índico. Curiosamente en el 2010 coincidiendo con el trabajo del primer registro de CTXs en Madeira [208] se publicó por primera vez un estudio en el que se mostró la distribución global y no local de *Gambierdiscus* [112]. Los análisis indicaron que cinco de las cepas analizadas son endémicas del Atlántico (incluyendo el Caribe, el oeste de India y el golfo de México), cinco son endémicas de la zona tropical del Pacífico, y que dos especies, *Gambierdiscus carpenteri* y *Gambierdiscus caribaeus* tienen una distribución global. Por lo que estas cepas podrían estar en otras áreas, no sólo en el océano Pacífico, Índico y mar Caribe.

En las especies *S. dumerili y S. fasciata* analizadas en el presente trabajo se confirmó la existencia de 3 CTXs típicas del Pacífico y una CTX de la zona Caribeña o Índica de peso molecular 1140,6 Da. La transferencia de las CTXs a través de la cadena alimentaria empieza en los dinoflagelados, después pasa a los peces herbívoros y luego a los peces carnívoros que se alimentan de los herbívoros. Tanto los peces herbívoros como los carnívoros pueden ser tóxicos ya que van acumulando las CTXs a medida que migran de unos lugares a otros. Se han descrito más de 400 especies de peces con CTXs [9] y una consideración general es que dentro de una misma especie y en una zona geográfica, los peces pequeños son menos peligrosos que los más viejos y grandes. Una vida útil más corta significa menos tiempo disponible para la acumulación de toxinas en el tejido de los peces. Los peces carnívoros y los situados en niveles tróficos más altos se pueden considerar más peligrosos que los situados en niveles inferiores (herbívoros). *S. dumerili* es una especie de peces considerados cazadores de gran alcance que se alimentan de otros peces e invertebrados y pueden ser bastante grandes, incluso llegan a pesar 70kg. Se han encontrado en el océano Atlántico, Pacífico y en las costas índicas y también en el mar Mediterráneo. La especie *S. fasciata* es característica del Atlántico occidental (desde Massachusetts hasta Brasil) y del Atlántico oriental (Madeira). Pero son peces invasivos y desde el 1993 se han registrado también en el Mediterráneo [225]. Estas especies se pueden mover de unas zonas a otras e ir acumulando la toxina. Además, la apertura del canal de Suez (1869) ha favorecido la invasión de especies marinas procedentes del océano Índico, a través del mar Rojo, que se han establecido recientemente en

el Mediterráneo [226,227]. También es importante destacar que algunas CTXs que se acumulan en el tejido de los peces, se pueden metabolizar en otros análogos diferentes, por ejemplo la CTX-1B y la 51-OH-CTX-3C proceden de CTX-3C y CTX-4B, respectivamente [9]. Estos dos análogos (CTX-1B y 51-OH-CTX-3C) son en última instancia los responsables de las intoxicaciones humanas porque son más tóxicos que las otras 2 CTXs [228,229].

A la teoría del cambio climático y que *Gambierdiscus* puede tener una distribución mundial hay que sumarle la introducción de aguas de lastre de los barcos y el incremento del tráfico marítimo que también contribuyen a la aparición de algunas floraciones tóxicas [10]. Como consecuencia de todos estos factores, las CTXs están apareciendo en las costas europeas y, a nivel de seguridad alimentaria, pueden llegar a suponer un elevado riesgo para la salud de los consumidores. Los resultados mostrados en la presente tesis doctoral también fueron tenidos en cuenta por EFSA para evaluar el riesgo de estas toxinas en la salud humana [203].

Además, en la identificación de CTXs, también existe la necesidad de buscar métodos para su detección ya que los síntomas de la intoxicación se utilizan para el diagnóstico delaCFPpero la confirmación de la misma se basa en la detección de las CTXs en la comida que queda o en plasma de pacientes intoxicados [230]. Por lo tanto, es importante disponer de métodos adecuados de cuantificación de CTXs para confirmar los casos de CFP. En el presente trabajo, la identificación del perfil de CTXs en las muestras de *S. dumerili* y *S. fasciata* se realizó por un sistema de UPLC-MS/MS. La columna que se empleó fue una C18 Waters Acquity UPLC BEH (100 x 2.1 mm, 1.7 µm) y una fase móvil compuesta de ACN y agua. El empleo de un sistema de UPLC-MS/MS permitió acortar mucho el tiempo del análisis para cada toxina (12 min) ya que hasta ahora la mayoría de los métodos descritos usan tiempos que superan los 50 min [73,108,109,198,199]. La detección de las CTXs también se realizó por un método *in vitro* obteniendo resultados comparables a los del método químico. Gracias a estos estudios y utilizando los estándares en los casos en los que disponíamos de ellos, se pudo obtener el perfil de CTXs en las dos especies: CTX-1B, CTX-3C, un análogo de peso molecular 1039,6 Da y una CTX de la zona caribeña o índica de peso molecular 1140,6 Da. Por lo tanto, los resultados son interesantes tanto desde el punto de vista de la identificación geográfica y del método de detección.

A lo largo de los años se ha hecho un gran esfuerzo en desarrollar métodos basados en la cromatografía líquida, no sólo para SPXs y CTXs, sino también para el resto de las toxinas lipofílicas. Los primeros métodos, se desarrollaron para detectar clases específicas de compuestos. A medida que se identificaban nuevos grupos de toxinas, se iban desarrollando los métodos de cromatografía líquida. Los últimos fueron para AZAs y SPXs ya que se descubrieron a mediados de los años 90 [231-233]. Cada método de cromatografía líquida empleaba distintas columnas cromatográficas, fases móviles y gradientes adaptados a las características y pesos moleculares de cada grupo de toxinas. Pero en la última década, se ha priorizado el desarrollo de metodologías dedicadas a detectar un gran número de toxinas lipofílicas utilizando un método de análisis de multi-toxinas [162,188]. Este método se desarrolló con el fin de utilizarse en los programas de monitoreo ya que en un mismo análisis y en un periodo de tiempo corto se puede detectar un gran número de toxinas. Después de que el método de multitoxinas ha sido evaluado por varios laboratorios que lo han considerado adecuado para el análisis oficial de toxinas lipofílicas, se ha convertido en método de referencia desde el 1 de julio de 2011 [164]. En este contexto, la tercera parte de la presente tesis doctoral consistió en la evaluación del método oficial que emplea la cromatografía líquida combinada con MS/MS para la detección de las toxinas lipofílicas [234]. El estudio consistió en evaluar si parámetros como la marca de disolventes, el número de toxinas incluidas en los métodos de MS o los equipos, pueden afectar a la cuantificación de estas toxinas. Es decir, condiciones que no están incluidas en la guía de referencia [189] por lo que cada laboratorio puede usar los que considere más adecuados. El estudio se hizo con el grupo de las toxinas diarreicas ya que es un grupo de toxinas reguladas y hay estándares disponibles comercialmente. En este estudio se observó que la cuantificación de OA, DTX-1 y DTX-2 se modifica al variar estos parámetros. Por ejemplo cuando estas toxinas se cuantifican por métodos de detección de MS que incluyeron diferente número de compuestos a monitorizar, las cantidades de cada toxina aumentaron o disminuyeron. Esto se observó con dos métodos de MS, uno que incluía las transiciones para las toxinas diarreicas (un total de 4) y el otro incluía las transiciones para 6 toxinas lipofílicas (un total de 10). Las cantidades analizadas usando el método con 10 transiciones fueron considerablemente menores que las obtenidas por el método que incluyó 4 transiciones. Las subestimaciones fueron de hasta un 40% para el OA, un 39 % para la DTX-1 y un 37 % para la DTX-2. Esto significa que el número de

transiciones incluidas en los métodos de MS, no fijadas en el método de referencia [189] afecta a la cuantificación de las toxinas. Cuando las toxinas no están disponibles comercialmente, la curva de calibración de un estándar se utiliza para cuantificar otras toxinas del mismo grupo asumiendo que dan el mismo factor de conversión [231]. Esta cuestión también se evaluó y no se obtuvieron resultados comparables. Cuando cada uno de los tres análogos se cuantificó usando la curva de los otros dos, las cantidades se vieron aumentadas o disminuidas, es decir se obtuvieron resultados erróneos en la cuantificación. La variabilidad más grande se obtuvo cuando DTX-2 se cuantificó con la curva de calibración de la DTX-1. En este caso la sobreestimación fue del 204%. Cuando DTX-2 se cuantificó usando la curva de calibración de OA, la sobreestimación fue del 88%. Este tipo de cuantificación es muy común ya que el estándar del OA está comercialmente más disponible que el de la DTX-2. Por lo tanto, el uso de estándares individuales para cada toxina es esencial para la exacta cuantificación de estos compuestos por MS y todas las toxinas para las que no existen estándares no se deberían de monitorizar en las muestras. Como era de esperar, este trabajo no pasó desapercibido para una parte de la comunidad científica que defienden las técnicas de MS como método de referencia para el análisis de toxinas lipofílicas. Este estudio dio lugar a tres publicaciones en la misma revista, *Analytical Chemistry*, donde otros grupos de investigación comentan y preguntan curiosidades acerca de este estudio [235-237]. Las respuestas y aclaraciones a estos comentarios se recogen en otra publicación realizada por nuestro grupo de investigación [238].

En resumen, los estudios de toxicidad llevados a cabo con los SPXs sugieren que se deberían de establecer límites legales para los SPXs. Las CTXs son muy tóxicas y si sigue aumentando su frecuencia en nuestras costas pueden suponer un problema de salud pública por lo que también deberían de establecerse límites y métodos oficiales para su detección. Si esto sucede, el método de referencia más probable para los SPXs es el LC-MS/MS puesto que ya se incluyen con el resto de las toxinas lipofílicas en los programas de monitoreo. No obstante, en la presente tesis doctoral, se propone un método funcional para su detección ya que uno de los principales problemas de los métodos químicos es la falta de estándares. Este problema aumenta con el grupo de las CTXs por la gran cantidad de análogos pertenecientes a este grupo y los pocos estándares disponibles. Hasta la fecha se han identificado más de 50 análogos [112] y es muy frecuente la aparición de varias

CTXs con el mismo peso molecular en la misma muestra que eluyen en diferentes tiempos de retención [104]. Por lo que sin poseer el estándar específico del compuesto es difícil su correcta identificación. Esta carencia de estándares junto con la pequeña cantidad de material contaminado disponible para el desarrollo de los métodos, la validación de la tecnología basada en la cromatografía líquida con detección por MS está muy limitada y, hasta el momento, no se han llevado a cabo estudios en distintos laboratorios. Se ha demostrado que la tecnología basada en la LC-MS/MS es extremadamente útil para identificar perfiles característicos de toxinas en diferentes matrices en todo el mundo [73,200] ya que muchas veces, las intoxicaciones se deben a distintas toxinas que tienen el mismo mecanismo de acción. Estos métodos son útiles para caracterizar nuevas toxinas y buscar análogos. En general, los LODs obtenidos por los métodos de MS son bajos [195,239] y las toxinas se detectan a niveles por dejado de los límites actualmente legislados. Estos métodos son específicos, sensibles y tienen la posibilidad de una detección multi-toxinas. Sin embargo a la hora de proteger la salud del consumidor se deberían de ajustar y definir más las condiciones del análisis así como determinar los factores de toxicidad.

5. Conclusiones

1. El dinoflagelado *Alexamdrium ostenfeldii* produce elevadas cantidades de los análogos 13-desmetil espirólido C y 13,19-didesmetil espirólido C cuando crece en medios de cultivo F2 o Walne, una salinidad del 28 ‰ y un fotoperíodo de 24h.

2. La medida de la unión directa de los espirólidos al receptor nicotínico por polarización de la fluorescencia es un método sencillo, sensible y rápido para cuantificar los análogos 13-desmetil espirólido C y 13,19-didesmetil espirólido C en muestras de mejillón.

3. Los espirólidos se absorben por vía oral y se detectan en sangre a los 15 minutos, en orina a la hora y en heces a las 24 horas de su administración.

4. Después de una administración intraperitoneal, el 13-desmetil espirólido C tiene una LD_{50} de 27,9 µg/kg, el 13,19-didesmetil espirólido C de 32,2 µg/kg, mientras que el análogo 20-metil espirólido G no muestra toxicidad.

5. Las elevadas cantidades de ciguatoxina-1B, ciguatoxina-3C y análogos detectadas en peces del género *Seriola* confirman el riesgo de la expansión de este grupo de toxinas por el sur de Europa.

6. El método de cromatografía líquida con detección por espectrometría de masas para cuantificar toxinas lipofílicas requerido por la legislación europea, no ofrece las garantías mínimas de seguridad puesto que los resultados pueden fluctuar hasta un 50%.

7. La cuantificación de una toxina lipofílica utilizando como calibrante otro análogo puede generar errores de hasta un 204% según el nuevo método oficial de detección. Por lo tanto, en este método es crítico fijar las condiciones de todas las variables para obtener resultados fiables.

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